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ENDOCRINOLOGY

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NUMBER 1

EFFECT OF CARBON TETRACHLORIDE-FEEDING ON ESTROGEN EXCRETION IN THE NORMAL FEMALE GUINEA PIG¹

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IN ORDER to confirm and extend previous investigations in which increases in urinary estrogens were found in rats after partial hepatectomy (Schiller and Pincus, 1944), and in humans who had chronic liver cirrhosis accompanied by gynecomastia (Glass, Edmondson and Soll, 1940, 1944) or acute hepatitis (Gilder and Hoagland, 1946), the total estrogen excretion has been studied in normal female guinea pigs before, during and after carbon tetrachloride feeding.

EXPERIMENTAL

Materials and Methods: Six female guinea pigs weighing an average of 815 g. at the beginning of the experiment were used. Urine was collected irregularly over a period of 164 days before the CCl_4 treatment was begun, over 141 days during CCl_4 feeding, over a 45-day period after cessation of CCl_4 feeding and over a 16-day period during which CCl_4 feeding was resumed. Each guinea pig was fed twice weekly with 0.4 ml. of a CCl_4 -Wesson oil mixture in which the CCl_4 content was varied from 0.02 to 0.08 ml. per dose. These dosage levels for CCl_4 were sublethal as compared with 100 per cent fatality obtained within seven days after one administration of 0.2 ml. CCl_4 .

Each urine sample was collected for 72 hr. periods over 25 ml. 12N HCl. The urine was filtered, hydrolyzed for 15 min. with 0.15 vol. 12N HCl and extracted with diethyl-ether. The ether extract was washed with H_2O and Na_2CO_3 and the total phenolic steroids were extracted with 2N NaOH, acidified and re-extracted with ether according to the method of Pincus (1945).

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² Ayerst, McKenna and Harrison, Ltd., Fellow.

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TABLE 1. AVERAGE VOLUME OF URINE AND AMOUNT OF ESTROGEN EXCRETED BY FEMALE GUINEA PIGS BEFORE, DURING, AND AFTER CCl_4 FEEDING

Sample number	Urine period start	Collection (days)* finish	Average volume urine per G.P. per day (ml.)	Number of doses of CCl_4	Total vol. CCl_4 (ml.)	Ave. mcg. estrogen	
						L. urine†	G.P./day‡
3	-143	-139	21.0			2.34	.0491
6	-107	-104	33.1			1.00	.0331
12	-38	-35	22.2			1.50	.0332
14	-3	0	25.5			0.95	.0243
Average Normal			21.0 \pm 6.6			1.45 \pm 0.64	.0349 \pm .0103
15	4	7	23.6	2	0.04	6.11	.144
16	11	14	23.3	4	0.08	10.00	.238
18	25	28	19.4	8	0.16	52.0	1.009
19	32	35	28.8	10	0.20	319.00	9.13
20	47	50	27.2	12	0.24	495.00	13.45
21	67	70	29.4	19	0.44	120.00	3.53
22	74	77	31.6	21	0.51	50.00	1.60
23	88	92	34.4	25	0.67	18.9	.649
24	102	106	28.8	29	0.86	4.61	.133
25	132	135	26.9	33	1.09	9.13	.247
26	141	145	19.5	36	1.33	6.33	.123
Average			26.9 \pm 4.5				
27	188	193	15.4			2.03	.0314
28	193	200	20.0			1.56	.0311
29	204	209	18.6			2.57	.023
Average			18.2 \pm 1.9				
30	209	214	15.3	1†	0.08	4.38	.0670
31	214	221	15.7	2†	0.16	12.5	.270
32	221	225	12.6	3†	0.24	15.3	.330
Average			17.6 \pm 2.7				

* 0 = day first dose of CCl_4 was given, and a negative number = days before CCl_4 feeding was begun.

† A second series of CCl_4 feedings.

‡ Estrogen expressed as mcg. estrone equivalents.

The ether was evaporated and the residue stored in a vacuum desiccator. For bioassay of the total estrogenic activity the residue was dissolved in sesame oil and administered intravaginally according to the method of Lyons and Templeton, 1935, on spayed female rats which had been standardized previously (Pugsley and Morrell, 1943). Smears were read according to Kahnt and Doisy (1928). All rats were primed with 0.15 mcg. estrone every 14 days and assays were carried out during the intervening weeks.³

Results: The results of the assays of the urine extracts, shown in table 1 and figure 1, have been correlated with the dose and time at which CCl_4 was administered, and with the volume of urine excreted. The estrogen excretion (expressed as mcg. equivalents of estrone) has been plotted as concentration per liter of urine and as amount per guinea pig per day, against time in days. The assays were calculated by the probit method and it was found that the average deviation for the method was 9.8 per cent. A standard deviation of 43.4 per cent was obtained for the estrogen concentration in the urine of normal animals while the standard deviation of the amount of estrogen excreted per animal per day was 26.8 per cent. This indicated that in normal animals total daily excretion was more constant than the urinary concentration.

³ Crystalline estrone was supplied by Ciba Pharmaceutical Products, Inc., Summit, N. J., and by Eli Lilly Co., Indianapolis, Ind.

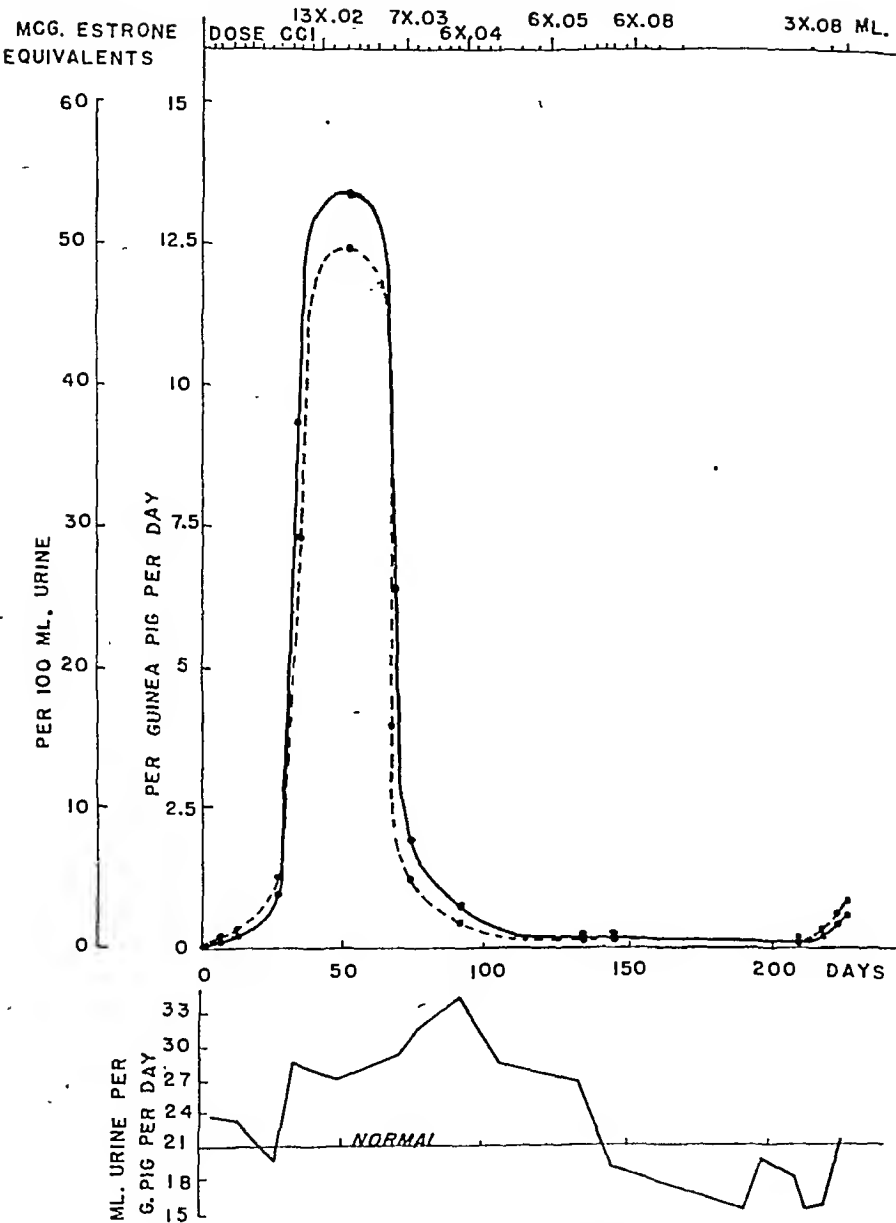


Fig. 1. Effect of CCl_4 Feeding on Estrogen Excretion and on Urine Volume of Female Guinea Pigs.*

* Estrogen excretion per guinea pig per day shown by solid line of upper curve; estrogen concentration per 100 ml. urine shown by broken line of upper curve. Figures arranged horizontal at top indicate size and number of doses of CCl_4 as well as time of administration.

Lower curve shows change in average volume of urine excreted per guinea per day during course of the experiment. Horizontal line in this graph indicates average, normal urine volume (approximately 21 ml. 1 g. pig/day).

The data in table 1 and figure 1 show significant changes in urine volume as a result of CCl_4 feeding. The mean volume of urine excreted by normal animals was 21.0 ml. per guinea pig per day, with a standard deviation of 6.6 ml.; the mean volume during 141 days of CCl_4 feeding was 26.9 ± 4.5 ml.; at the end of the recovery period, the mean volume was 18.2 ± 1.9 ml.; a further decrease to 17.6 ± 2.7 ml. occurred when CCl_4 feeding was resumed.

It will be noted that when CCl_4 was fed to normal guinea pigs the excretion of endogenous estrogenic substances showed three distinct stages: (1) The estrogen excretion increased during the first 50 days, at first slowly and then rapidly, represented by a sigmoid curve. This was accompanied by an initial fall in the volume of urine which returned to a level significantly above normal during the period of strikingly elevated estrogen excretion. The excretion of estrogen per day increased at a faster rate than the estrogen concentration in the urine until the peak was reached (except for a lag during the first 25 days). The estrogen excretion at the peak of the curve was approximately 350 times the normal value. (2) The estrogen excretion decreased during the second 50 days, at first rapidly and then slowly, also represented by a sigmoid curve. The total estrogen excretion decreased more slowly than the concentration until the 88th day. (3) The estrogen excretion was relatively constant at a level 3.7 times normal throughout the remaining 50 days of CCl_4 feeding.

It will be noted that the slope of the daily excretion curve between any two points, compared to the slope of the concentration curve between corresponding points, always was less in the presence of a decrease in urine volume and greater in the presence of an increase in volume except between days 88 and 141 (see fig. 1). At the end of the period during which no CCl_4 was fed (to permit recovery), both the concentration and total output of estrogen decreased, so that they fell within the normal range. The greater drop of the total estrogen excretion again was coincident with further decrease in urine volume. On administering a second series of CCl_4 feedings, it was interesting to note that, in spite of pre-existent liver damage, the effect should so closely duplicate that of the first series, both in the fall of urine volume and the increase in estrogen excretion, at least for the short period covered by this second series of CCl_4 feedings.

Autopsy of two animals at the end of the recovery period showed the vaginae to be closed and previously noted edema of the subcutaneous tissues had disappeared almost entirely. No adhesions or fibroids were found in the abdominal cavity. The livers showed advanced secondary cirrhosis, with no signs of recovery. They were dark red with a "lemon rind" appearance. The ovaries were enlarged and numerous follicles were present. The adrenals were hemorrhagic and only slightly, if at all, enlarged.

DISCUSSION

Estrogen Excretion in Normal Female Guinea Pigs

Emmens (1943) determined estrogen excretion in the normal female guinea pig and found 377 ± 80 "effective intravaginal mouse doses" excreted per guinea pig per five days, with a range from 90 to 600 effective doses. He defined one effective intravaginal mouse dose as 0.0029 mcg. of estrone. This is equal to $.0218 \pm .0046$ mcg. per guinea pig per day with a range from .0052 to .0348 mcg. The average excretion of $.0349 \pm .0103$ mcg. per guinea pig per day (range from .0234 to .0491 mcg.) reported here compared with the values obtained by Emmens, shows that our results were higher but the average falls within the upper limits of Emmens' range. His average value is only slightly below the range reported by us. This discrepancy may be due in part to a difference in age, strain or nutritional state of the two groups of animals or to a difference in the method of extraction, since he used benzene which is less efficient than ether. In addition, the use of the mouse instead of the rat as the assay animal has been shown to give lower values and less accurate results (Hain and Robson, 1936, and Curtis, Witt and Knudsen 1944).

Beerstecher (1942) found that the average volume of urine excreted by virgin female rabbits varied inversely with the concentration of estrogen. He reported an average of 30 ml. per rabbit per day from August to May when the estrogen concentration was 1.0 mcg. per liter, and 20 ml. per day from May to August when the concentration was 2.0 mcg. per liter.⁴ In our studies with the normal female guinea pigs the inverse ratio of estrogen concentration to volume of urine excreted was observed also and was more constant than either the direct or inverse ratio of total estrogen per day to urine volume. Thus, the product (concentration \times volume), which is the total estrogen excretion per day, tends to approach a constant value.

Effect of CCl₄ Administration on Estrogen Excretion

(1) Estrogen excretion during first 50 days: The increased excretion of estrogen during the first 50 days of CCl₄ feeding agrees with the findings of Talbot (1939) and of Pincus and Martin (1940) who reported increased activity of endogenous and exogenous estrogens after CCl₄ administration to rats. Talbot found the uterine weight of rats fed CCl₄ was increased above normal while the weight of uteri of ovariectomized rats did not increase with CCl₄ administration. Pincus and Martin found a given dose of estrogen was 80 per cent more

⁴ It is surprising that the volume of urine excreted per day by the rabbits reported by Beerstecher is only slightly more than the volumes excreted per day by our guinea pigs.

effective in causing vaginal estrus after CCl_4 administration to rats than in nontreated animals. It should be pointed out, however, that in both experiments CCl_4 was administered for only short periods of time and then only slightly sublethal doses were used, and that the effects of CCl_4 on estrogen excretion were not determined.

It has been demonstrated also that any impairment of normal liver function results in an enhanced effect of both endogenous and exogenous estrogens. Such impairment has been obtained by: 1) vitamin B complex deficiency diets in rats by Biskind and Biskind (1941, 1942), Biskind and Selesnyak (1942), Segaloff and Segaloff (1944), in mice and rats by Szego and Barnes (1943), and Shipley and Gyorgy (1944); 2) inanition in rats by Shipley and Gyorgy (1944) and Drill and Pfeiffer (1946); 3) partial hepatectomy in rats by Schiller and Pincus (1944) and Roberts and Szego (1947); 4) chronic liver cirrhosis with gynecomastia in men by Glass, Edmondson and Soll (1940, 1944); and 5) acute infectious hepatitis in men by Gilder and Hoagland (1946).

The reduction of estrogenic effect has been localized to the liver *in vivo* by reports of a greater effect of systemic as compared to portal introduction of both endogenous and exogenous estrogens (Evans and Burr, 1922; Golden and Sevringhaus, 1938; Biskind and Mark 1939; G. R. Biskind, 1941a, 1942; Segaloff and Nelson, 1941; Segaloff, 1943; Engel, 1944). Further evidence that the liver is the site of estrogen inactivation has been obtained by the marked decrease in estrogenic activity 1) after perfusion of estrogen through the liver of dogs (Israel and Meranze, 1937) and rats (Schiller, 1945), and 2) after *in vitro* incubation of estrogen with liver tissue of dogs (Silberstein, Molnar and Engel, 1933 and Zondek, 1934), rats and rabbits (Heller, Heller and Sevringhaus, 1939, Heller, 1940) and guinea pigs in different sexual states (Engel, 1941).

Localization of estrogen inactivating ability to the hepatic cells rather than the Kupfer cells was obtained by Zondek and Sklow (1941) who inhibited the reticulo endothelial system with colloidal copper. That the inactivation is enzymatic is indicated by the work of Zondek (1934) who found that liver tissue lost its inactivating ability after heating, by Heller (1940) who reported inhibition of inactivation by NaCN and by Engel (1945) who obtained inactivation with an aqueous extract of beef liver, the active principle of which could be concentrated by alcoholic precipitation. However, Cantarow, Paschik, Rakoff and Hansen (1943) were not able to find any difference in ability to inactivate estradiol between normal rat liver tissue and liver from animals fed with CCl_4 . Their findings suggest that some mechanism other than enzyme inactivation may be responsible for the enhanced estrogenic activity *in vivo* following administration of CCl_4 .

In view of the evidence cited above, it seems probable that the in-

Increased estrogenic activity obtained in the urine when CCl_4 is fed to normal female guinea pigs may be due to an impairment of the ability of the liver to inactivate these hormones. Two stages of increased estrogen excretion are present, the first at a slow rate accompanied by a decreased urine volume (day 0 to day 14), the second a sharp increase accompanied by larger urine volume (day 14 to day 50). It is likely that the ability of the liver to convert the more active estrogens to estriol is lost first, since conversion of estrone to estriol is reduced in hepatectomized rats (Sehiller and Pineus 1944). Following this, loss of interconversion between estrone and estradiol presumably would leave only the most active estrogen, estradiol, since this is the compound reportedly formed by the ovary. Since CCl_4 is known to be a nonspecific toxic agent and since other tissues than the liver are capable of this conversion in the normal animal (Heller, 1940) it is possible that the magnitude of the increased estrogen excretion obtained in our experiments may be due to an impairment of this mechanism, not only in the liver, but in other tissues as well. The marked changes in the average volume of urine excretion during the course of this experiment suggests a change in kidney function that may be due, directly or indirectly, to the cytotoxic action of CCl_4 .

(2) Estrogen excretion from day 50 to day 145: The decrease in estrogen excretion, following the initial increase, has not been reported previously to our knowledge. Moreover, a peak of estrogen excretion as great as 350 times the normal level also has not been reported previously with endogenous estrogens as the only source. The use of more severe experimental procedures, carried out for a relatively short time, may be responsible for the failure of previous investigators to obtain these results. In the studies using hepatotoxic agents only a few relatively massive doses were administered. For example Pineus and Martin (1940) used 0.1 ml. CCl_4 three times a week in rats while we used only 0.02 to .08 ml. twice a week in guinea pigs. Thus, the dose/body weight ratio was much smaller in our experiments. In studying the effect of vitamin B complex deficient diets and of inanition (described above) the experiments extended over a total of only 35 to 60 days with no evidence of enhanced estrogen activity during the first 30 days, while CCl_4 fed animals showed an immediate increase in estrogen excretion but the peak, followed by decrease in excretion, was not reached until 50 days after such treatment. It is possible that if the dietary experiments had been continued for a longer time a peak of activity, followed by regression, might have been observed. The absence of histologic change in the liver during the first few weeks with certain types of dietary deficiency as compared to severe hepatic damage, even within the first few days of CCl_4 feeding, indicates the former to be a less severe process. When hepatectomy is used to damage the liver, the acute decrease in functioning liver

tissue, followed by rapid regeneration, may preclude the production of secondary changes in other organs that are found when chronic liver damage is maintained by repeated assaults of sublethal doses of CCl_4 .

The marked decrease in estrogen excretion observed between day 50 and day 100 suggests several possibilities 1) that estrogens are not being produced because of damage by CCl_4 to the pituitary or the ovaries, 2) that activation of the estrogens by the liver does not occur (Szego and Roberts 1946), or 3) that inactivation of estrogens is resumed either by the liver or by some other organ.

(3) Recovery from day 145 to day 209: When the effects of extended CCl_4 administration are allowed to regress for 45 days, both the total daily excretion and urinary concentration of estrogen return to the normal range (although urine volumes were significantly lower than normal). Advanced secondary cirrhosis was present in the livers and showed no signs of recovery. This observation and those of Cantarow, *et al.* (1943) suggest that normal liver morphology need not be necessary for estrogen inactivation and that physiological repair (at least a return of the function of estrogen inactivation) may precede morphological repair in the liver.

Effect of CCl_4 Feeding After Recovery (day 209 to 225)

When CCl_4 feeding was resumed after a 45-day period of rest, an increased estrogen excretion and decreased urine volume occurred as it did following the initial CCl_4 feedings. This duplication of the original findings suggests that the function of inactivation returns toward normal soon after cessation of treatment with the toxic agent.

Probably no one single hypothesis is adequate to explain the effects of CCl_4 feeding on estrogen excretion, but rather, they are due to a series of factors produced by, 1) direct cytotoxicity of CCl_4 , and 2) the indirect effect of functional impairment by CCl_4 on interdependent processes.

SUMMARY

By the use of the intravaginal assay method it is found the normal female guinea pig excretes $0.035 \pm .010$ mcg. estrone equivalents per day with a range from 0.023 to 0.049. When CCl_4 is fed, by the method and dosage described, the following changes in estrogen excretion occur (expressed as mcg. estrone equivalents):

- (1) During the first 50 days the level of estrogen excretion increase to a peak 350 times normal.
- (2) During the second 50 days, the excretion level decreases, a phenomenon not reported previously.
- (3) During the third 50 days, a fairly constant level of estrogen excretion is maintained at approximately 3.7 times normal.

When the effects of extended CCl_4 feeding are allowed to regress for 45 days the levels of estrogen excretion return to normal. Resump-

tion of CCl_4 administration results in an increase in estrogen excretion similar to that following initial CCl_4 feeding.

It is suggested that the sequence of effects of CCl_4 administration are 1) an impaired inactivation of estrogens resulting in increased excretion, followed by 2) either an inhibition in the production of endogenous estrogens, or, loss of activation of estrogens resulting in decreased estrogen excretion.

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THE DETERMINATION OF ADRENOCORTICAL STEROIDS IN HUMAN URINE¹

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INTRODUCTION

VARIOUS methods for the determination of adrenocortical steroids in human urine have been proposed in recent years. Hormonally active corticosteroids have been measured by bioassay (e.g., Selye and Schenker 1938, Eggleston *et al.* 1946, Venning *et al.* 1946), but the relatively small concentrations of active material, particularly in normal human urine (Venning and Kazmin 1946, Heard 1948) has required the processing of large amounts of urine. The probability of the presence in urine of typical adrenocortical substances lacking biological activity has led to attempts at their measurement. Advantage has been taken of the fact that certain typical adreno-corticosteroids possess significant reducing activity by virtue of an α -ketol side chain at C-17 (Talbot *et al.* 1945) as well as the α, β unsaturation in ring A (Heard and Sobel 1946). In addition fairly specific determination of certain types may be had by periodic acid oxidation which generates formaldehyde from either α -ketol or glycol side chains (Lowenstein *et al.* 1946, Daughaday *et al.* 1948). In this paper we will compare the results of assay of reducing with formaldehydogenic activity.

METHODS

The urine for assay was collected from normal, healthy men and women of various ages. Two types of collection were made. The first involved three collections per 24 hours covering the periods of sleep, early morning, and rest of day (Pincus 1945). This type of collection permits the assessment of the diurnal rhythm of excretion of certain urinary steroids (Pincus *et al.* 1948a). The second consisted of three specimens from subjects in the basal state covering (a) a period (called prestress) of two hours preceding the administration of 50 mgm. equivalent of the Armour standard of pituitary adrenocorticotrophin² (ACTH), (b) a period (labelled stress) of one hour and fifteen

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minutes following ACTH administration, and (c) a period (labelled post-stress) of two hour following the stress collection.

The urines were extracted by the method of Heard and Sobel (1946) to yield a neutral lipid fraction. Aliquots were then made for further processing or colorimetric assay. The neutral lipid extract of certain urines was partitioned between benzene and water by the method of Talbot *et al.* (1945) to give a crude "C" fraction containing all benzene-soluble neutral lipid which is then extracted with water to give "benzene" and "water" fractions.

A separation of alcoholic lipids from the non-alcoholic constituents was effected by the method of Pincus and Pearlman (1941).

Determination of reducing lipid were made by the phosphomolybdic acid method (PRL) (Heard and Sobel 1946) and the copper reduction method (CRL) of Talbot *et al.* (1945). Formaldehydogenic lipid (FL) was determined by a 20-minute periodic acid oxidation followed by colorimetric measurement of the distilled formaldehyde generated (cf. Daughaday *et al.* 1948). All determinations are expressed as equivalents of a desoxycorticosterone standard.

RESULTS

Phosphomolybdic acid reducing lipids and formaldehydogenic lipids

In Tables 1 and 2 we present the data on diurnal urine collections from 34 men and 34 women, respectively, giving desoxycorticosterone equivalents of the neutral reducing lipids (PRL) determined by the Heard-Sobel method and of the formaldehyde generating lipids (FL). The latter method invariably gives lower values than the former. In both sets of data the tendency for individual morning and day values to exceed the sleep values is evident, as is the tendency for the men to excrete more reducing lipid, on the average, than the women.

In Tables 1 and 2 we present the mean output values (and their standard errors) and mean percentage change of the morning and day values over the sleep values. It should be pointed out that the mean percentage change is an average of the individual percentage changes which are not shown in the table. By the "t" test the data for men exhibit significantly higher absolute outputs (mgm. per 24 hours) of neutral reducing lipids in both the morning and the day values over the sleep values, while the data for the women do not. Statistically significant increase in FL is obtained in both groups for the morning increase over sleep but not for the day over sleep. When we consider the mean percentage increases of the morning and the day values over the sleep values all changes are significant for both men and women. As we have pointed out elsewhere (Pincus *et al.* 1949) taking the percentage changes tends to bring together the data for individuals having different characteristic absolute levels of output. We may therefore conclude that the data for both the men and women in this series exhibits a diurnal rhythm. Such a diurnal rhythm has been previously noted for neutral reducing lipids in men (Pincus *et al.* 1948a).

It should be pointed out that although the formaldehydogenic

TABLE 1. NEUTRAL REDUCING LIPID (PRL) AND FORMALDEHYDOGENIC LIPID (FL). URINARY OUTPUTS OF NORMAL, HEALTHY MEN

Subject No.	Age Yrs.	Mg. per 24 hours					
		PRL*			FL*		
		Sleep	Morning	Day	Sleep	Morning	Day
1	23	3.58	2.90	3.26	0.32	0.46	0.34
2	23	3.78	3.76	1.98	0.69	0.50	0.35
3	26	1.85	1.87	2.76	0.34	0.39	0.54
4	28	3.16	5.93	4.77	0.34	0.72	0.48
5	29	2.03	1.97	2.56	0.30	0.44	0.29
6	32	1.91	3.13	2.43	0.30	0.55	0.36
7	32	2.92	5.60	3.19	0.39	0.36	0.35
8	34	1.42	2.00	2.42	0.17	0.30	0.28
9	35	2.31	3.76	2.48	0.44	0.64	0.30
10	36	1.49	2.50	2.32	0.27	0.48	0.30
11	37	1.28	1.62	1.80	0.55	0.21	0.91
12	39	1.82	1.42	2.33	0.22	0.24	0.30
13	41	2.14	3.66	1.35	0.35	0.82	0.31
14	42	2.76	3.48	1.57	0.32	0.41	0.29
15	45	2.40	2.52	4.20	0.14	0.28	0.30
16	46	1.74	2.75	2.65	0.29	0.40	0.39
17	47	1.79	1.15	1.75	0.13	0.29	0.22
18	47	1.52	1.80	2.64	0.22	0.30	0.34
19	47	2.38	2.61	2.44	0.31	0.47	0.37
20	47	2.56	3.12	2.80	0.35	0.58	0.50
21	47	2.49	1.90	2.88	0.20	0.19	0.25
22	49	2.50	3.43	3.01	0.41	0.61	0.45
23	51	1.99	—	2.16	0.65	—	0.34
24	52	1.32	2.33	1.91	0.17	0.63	0.40
25	52	1.30	2.10	3.48	0.28	0.36	0.54
26	52	2.23	2.78	4.28	0.60	0.48	0.70
27	54	1.60	1.46	1.99	0.22	0.31	0.32
28	55	1.91	2.38	1.75	0.24	0.31	0.21
29	61	1.24	1.75	1.84	0.19	0.30	0.14
30	61	1.73	1.45	1.71	0.10	0.31	0.13
31	61	1.84	2.52	2.16	0.27	0.38	0.23
32	63	1.92	2.34	3.28	0.21	0.40	0.27
33	76	1.46	3.34	1.72	0.24	0.22	0.18
34	80	3.41	1.17	1.50	0.79	0.34	0.29
Means †		2.11 ±	<u>2.62 ±</u>	<u>2.51 ±</u>	0.32 ±	<u>0.41</u>	0.35
Mean % increase } over sleep †		0.115	<u>0.193</u>	<u>0.140</u>	0.027	<u>±0.026</u>	<u>±0.026</u>
		—	<u>28 ± 7.3</u>	<u>27 ± 7.5</u>	—	<u>54 ± 11.9</u>	<u>23 ± 8.2</u>

* Expressed as 11-desoxycorticosterone equivalent.

† Numbers underlined with a single line give a "t" value for difference from the sleep determinations @ the 2% to 5% level of confidence, those underlined with a double line give a "t" value @ the 1% level of confidence or below.

lipid shows a greater net increase in the morning in both sexes than does the neutral reducing lipid (col. 3) the percentage increases are nonetheless not significantly different for the men's data ($t=1.87$, $p>.05$) but are for the women's data ($t=2.38$, $p<.05$). In brief, the increase in FL observed in the morning hours are larger than the increases of PRL, but they are more variable and so are of questionable significance. That the variability of the PRL method is less than the FL method is obvious from the coefficients of variation of the data. For instance, the FL coefficients of variation for the sleep determina-

TABLE 2. NEUTRAL REDUCING LIPID (PRL) AND FORMALDEHYDOGENIC LIPID (FL). URINARY OUTPUTS OF NORMAL, HEALTHY WOMEN

Subject No.	Age Yrs.	Mg. per 24 hours					
		PRL*			FL*		
		Sleep	Morning	Day	Sleep	Morning	Day
1	22	1.70	1.70	1.34	0.46	0.43	0.15
2	22	1.47	2.16	3.79	0.19	0.46	0.47
3	23	2.38	2.26	2.35	0.23	0.43	0.35
4	26	1.44	1.94	2.12	0.82	0.71	0.68
5	26	1.31	1.21	1.45	0.33	0.47	0.36
6	29	1.54	1.95	1.60	0.31	0.23	0.62
7	31	2.24	2.60	3.17	0.39	0.38	0.18
8	31	0.92	1.49	1.34	0.22	0.91	0.43
9	32	1.06	1.10	1.35	0.27	0.65	0.56
10	32	1.20	2.07	2.00	0.57	0.98	0.59
11	33	2.29	3.42	4.13	0.67	0.95	0.86
12	33	1.20	2.09	1.55	0.24	0.74	0.65
13	36	1.61	1.57	1.57	0.50	0.44	0.29
14	39	1.61	1.76	2.10	0.45	0.76	0.54
15	41	1.05	1.50	1.00	0.40	0.57	0.41
16	43	1.29	1.80	1.55	0.31	0.80	0.69
17	47	1.09	1.63	2.60	0.35	0.60	0.74
18	48	1.37	1.43	1.55	0.56	0.84	0.50
19	49	2.31	2.13	2.08	1.02	1.15	0.83
20	50	1.25	1.35	0.59	0.47	0.88	0.33
21	52	1.17	1.65	0.82	0.55	0.68	0.47
22	54	0.97	1.14	0.96	0.50	0.65	0.44
23	56	3.02	2.18	1.54	0.82	0.48	0.42
24	58	1.63	1.43	2.14	0.31	0.57	0.88
25	59	1.60	—	1.80	0.37	—	0.33
26	60	2.70	1.52	1.37	0.83	0.41	0.23
27	63	0.92	0.95	1.10	0.12	0.24	0.24
28	68	0.73	0.71	0.75	0.13	0.07	0.08
29	69	2.65	1.80	2.12	0.32	0.26	0.21
30	69	1.53	0.61	2.15	0.21	0.31	0.24
31	69	0.82	1.28	1.04	0.26	0.64	0.25
32	75	0.80	1.95	1.51	0.10	0.12	0.10
33	81	1.01	1.97	1.21	0.12	0.45	0.45
34	89	1.06	1.18	1.06	0.18	0.19	0.13
Means†		1.48±	1.65±	1.75±	0.40±	0.56±	0.43±
Mean % increase over sleep†		0.105	0.097	0.136	0.039	0.045	0.038
		—	<u>21±7.2</u>	<u>21±8.1</u>	—	<u>61±14.9</u>	<u>30±13.8</u>

* Expressed as 11-desoxycorticosterone equivalent.

† Numbers underlined with a single line give a "t" value for difference from the sleep determinations @ the 2% to 5% level of confidence, those underlined with a double line give a "t" value @ the 1% level of confidence or below.

tions of mean and women respectively are 50.6% and 57.0%; the corresponding PRL coefficients are 31.8% and 41.2%.

In a previous paper (Pincus *et al.* 1948a) we reported that although the neutral reducing lipid (PRL) and 17-ketosteroid excretion of men both exhibited a diurnal rhythm the *changes* in diurnal excretion of the two did not correlate significantly, suggesting that the secretion of the precursors of each might be under different influences. Furthermore absolute levels of output of each correlated only in the morning urine collections. Accordingly we have examined the relationship be-

tween the excretion of neutral reducing lipids and formaldehydogenic lipids. The correlation coefficients of these determinations for each collection period as well as the correlation coefficients for the percentage changes in morning and day collections have been determined, and in each set of data positive correlation coefficients are obtained. They vary between +0.21 and +0.62, and only one (+0.21 for the percentage change of morning output in the men's data) falls above the 5% level of confidence. We take these positive correlations to signify that formaldehydogenic and neutral reducing lipids vary quantitatively in the same way and that the diurnal changes also are similar for both.

In view of the large range of ages exhibited in the subjects of this study, we have examined the data for any notable relationship between age and the excretion values obtained. Dividing the men into 23-45 and 46-80 year groups gives no significant difference of output of either PRL or FL; a similar division of the women into 22-45 and 46-89 year age groups reveals no consistent difference (cf. Pincus *et al.* 1946). Nor do the positive correlations between PRL and FL disappear when the data for these age groups are examined. The implication is, therefore, that ageing does not differentially affect PRL or FL excretion.

The benzene:water partition of neutral lipids

To study further the possibility of dissociation of formaldehydogenic and neutral reducing lipid excretion various types of urine collections were partitioned between benzene and water as described above. The data on the fractions obtained are presented in Table 3. It is notable that the partitioning procedure results regularly in the

TABLE 3. THE NEUTRAL REDUCING LIPID (PRL) AND FORMALDEHYDOGENIC LIPID (FL) OUTPUTS IN BENZENE: WATER PARTITIONS

Experiment No.	Period of collection	Neutral reducing lipid mg. per 24-hrs. in		% of combined fraction in		FL mg. per 24-hrs. in		% of combined fraction in	
		1 Crude "C" fraction	2 Combined benzene: water fraction	3 Benzene	4 H ₂ O	5 Crude "C" fraction	6 Combined benzene: water fraction	7 Benzene	8 H ₂ O
1	20.17 hrs.	1.83	1.41	50	50	0.20	0.18	39	51
2	24.00 hrs.	0.53	0.51	58	42	0.13	0.08	40	60
3	Night	—	2.30	62	38	0.39	0.28	46	54
	Morning	—	1.52	67	33	0.59	0.48	52	48
	Day	1.09	1.02	60	40	0.22	0.17	47	53
4	Prestress	2.67	2.30	74	26				
	Stress (ACTH)*	2.74	2.17	63	37				
	Poststress	2.43	1.95	65	35				
5	Prestress	2.84	3.04	65	35				
	Stress (ACTH)*	4.20	3.73	72	28				
	Poststress	3.22	2.68	68	32				

* 50 mg. adrenocorticotrophic hormone (ACTH) administered at beginning of stress period.

loss of both neutral reducing lipid (compare cols. 1 and 2) and formaldehydogenic lipid (compare cols. 5 and 6). Accordingly, we have expressed the partitioning of each into the benzene and water fractions as percentages (cols. 3, 4, 7 and 8) of the sum (cols. 2 and 6) of the two fractions. In different urine samples 50% to 74% of the neutral reducing lipid activity tends to segregate into the benzene fraction (col. 3) whereas 39% to 52% of the formaldehydogenic lipids are found in this fraction (col. 7). The data of Table 3 do not, however, indicate any notable departure from the typical partitioning in different types of urine collection. For example, the PRL excreted after ACTH administration (experiments 4 and 5) does not partition differently from the prestress PRL. Nor does the partition of the formaldehydogenic substance in the diurnal samples of experiment 3 vary significantly with time of collection.

TABLE 4. SOME VALUES FOR OUTPUT OF NON-ALCOHOLIC REDUCING MATERIAL (PRL) PRESENT IN URINARY LIPID EXTRACTS

Experiment No.	Period	Mg. per 24-hrs. of total reducing lipid	Mg. per 24-hrs. of non-alcoholic reducing lipid	Percent of non-alcoholic in total reducing lipid
1	Night	1.39	0.33	24%
2	Prestress	1.71	0.52	30%
	Stress (ACTH)*	—	0.58	—
3	Prestress	1.76	0.37	21%
	Stress (ACTH)*	2.03	0.36	18%
4	Prestress	1.80	0.25	14%
	Stress (ACTH)*	3.52	0.62	18%
5	Desoxycorticosterone control	total 500 μ g.	56 μ g.	11%
6	Desoxyeorticosterone control	total 320 μ g.	30 μ g.	9%

* 50 mg. adrenocorticotrophic hormone (ACTH) administered at beginning of stress period.

Non-alcoholic reducing lipids

A portion of benzene-soluble reducing lipid may be due in part to substances which are not typical adrenal cortex steroids. This is suggested by the fact that the FL tends to partition in larger amount into the aqueous extract which should concentrate the more highly oxygenated corticosteroids (Talbot *et al.* 1945). The steroids possessing typical adrenocortical activity are all alcholic substances by virtue of the C₂₁ OH group. Accordingly they should be extractable as alcohols by the succinic anhydride reaction we have employed. In Table 4 we

present the reducing lipid (PRL) activity of the neutral non-alcoholic fractions of certain urine specimens. It will be noted that with amounts of desoxycorticosterone roughly comparable to the equivalents used in the urine aliquots approximately 10% failed to be extracted by the alcohol reagent (experiments 5 and 6) whereas larger proportions were found in this fraction after extraction of the urine samples (experiments 1 to 4). On the basis of the desoxycorticosterone control data, roughly 4% to 20% of the urinary fractions are probably non-alcoholic. Reducing activity of non-alcoholic substance in the phosphomolybdic acid reaction might be attributed to α,β unsaturated steroid ketones (Heard and Sobel 1946) or to miscellaneous non-steroidal substances. In any event it presumably contributes only a minor portion of the total reducing lipid activity, and shows no notable relation to ACTH stimulated output (experiments 2 to 4).

Phosphomolybdic acid and cupric ion reduction with various urinary extracts

Since the α,β unsaturation of certain typical steroids may be responsible for part of the reducing lipid values obtained by the Heard-Sobel method, opportunity for measurement of the reducing activity of the α -ketol side chain is offered by a study of the copper-reducing ac-

TABLE 5. PHOSPHOMOLYBDIC ACID REDUCING LIPID (PRL) AND CUPRIC ION REDUCING LIPID (CRL). URINARY OUTPUTS OF 16 WOMEN AND 2 MEN

Subject No.	Age in years	Mg. per 24 hours*					
		Phosphomolybdic acid reducing lipid*			Cupric ion reducing lipid*		
		Sleep	Morning	Day	Sleep	Morning	Day
1	32	1.51	0.97	4.13	0.45	0.66	1.19
2	55	1.27	1.33	1.90	0.45	0.80	0.46
3	56	2.72	2.10	1.30	1.18	0.57	0.30
4	61	0.79	1.13	1.20	0.31	0.40	0.36
5	62	1.43	1.62	1.85	0.61	0.38	0.35
6	67	1.41	2.52	3.00	0.32	0.37	1.05
7	70	1.34	1.30	1.60	0.90	0.59	1.05
8	72	1.39	2.49	1.80	0.51	0.86	0.58
9	76 (♂)	1.76	0.77	1.08	0.27	0.37	0.08
10	76	1.28	0.96	1.21	0.34	0.34	0.37
11	76	1.17	3.73	1.94	0.28	0.43	0.30
12	78 (♂)	0.82	—	1.74	0.27	—	0.33
13	79	1.25	3.04	2.84	0.42	1.11	0.60
14	82	0.74	0.68	0.72	0.30	0.36	9.24
15	82	1.62	2.20	2.80	0.45	0.74	0.53
16	83	2.84	2.10	1.21	0.71	0.46	0.59
17	90	1.13	1.20	1.38	0.80	0.45	0.27
18	91	1.51	0.95	0.98	0.19	0.14	0.21
Means†		1.44 ±	1.71 ±	1.82 ±	0.49 ±	0.53	0.49
Mean % increase†		0.132	0.376	0.251	0.062	±0.058	±0.075
over sleep values }		—	18 ± 17.7	38 ± 14.3	—	21 ± 13.7	14 ± 17.7

* Expressed as 11-desoxycorticosterone equivalent.

† Underlining as in Tables 1 and 2.

tivity of the neutral urinary lipids. Heard and Sobel (1946) have demonstrated that cupric ion reduction by the Talbot *et al.* method is not effected by α,β unsaturated ketones lacking the α -ketol side chain at C₁₇.

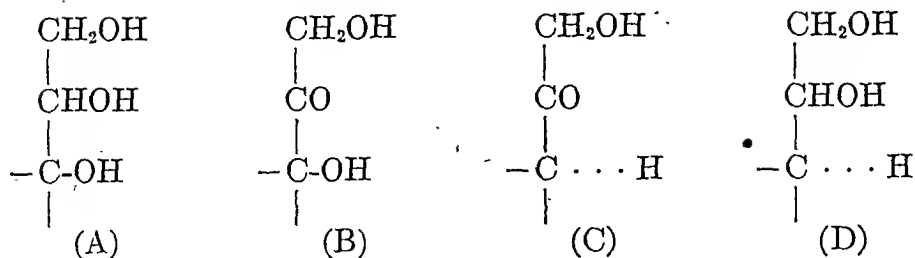
In Table 5 we present the data obtained on aliquots of diurnal rhythm urines measured by the phosphomolybdic acid and copper reagents respectively. Inspection of this table reveals consistently lower desoxycorticosterone equivalents by the latter method. In twelve of the eighteen subjects one or both of the non-sleep values exceeded the sleep values; this is true for fourteen of the eighteen in the CRL measurements. Increases are in the mean outputs for both PRL and CRL exhibited consistently for the morning and day values, but a significant percentage rise is had with these data only in the day values of the PRL measurements. It should be noted that the data of this table are contributed to chiefly by persons in the latter decades of life when there is ordinarily a less marked diurnal rhythm (Pincus *et al.* 1948b). The coefficients of variation are lowest for the PRL data in most of these measures, indicating a somewhat lower variability in the PRL method. Correlation coefficients between the CRL and PRL data of Table 5 have been calculated and are all positive (+0.25 to +0.78 in various measures), and all save one significant at the 1% level of confidence or below. This suggests that the same types of substances are measured by each method and that the output change of one tends to follow the output change of the other.

We have also measured the reducing lipid by these two methods before and after ACTH administration to three normal, healthy men. Both show corresponding increases: the mean PRL values were 1.62 and 2.78 mgm. desoxycorticosterone equivalents per 24 hours before and after ACTH respectively; the corresponding CRL values were 0.66 and 0.97. For the two methods these represent 72% and 52% output increases ascribable to the ACTH administration.

DISCUSSION

We believe that these data are notable for the demonstration of the entirely comparable output changes of the presumed urinary corticoid substance measured by the three methods employed. The values obtained by each method exhibit similar diurnal fluctuations and similar changes after ACTH administration. Furthermore, fairly consistent correlation of absolute output values is accompanied by remarkably consistent correlations between relative output changes. This is in contrast to the lack of correlation between 17-ketosteroid and neutral reducing lipid excretion (Pincus *et al.* 1948a).

Formaldehyde is generated from 20-21 α -ketols or glycols. Among the adrenal cortex steroids this would include substances having the following side chains at C₁₇:



Reduction of cupric ion is accomplished by side chains of types B and C and reduction of phosphomolybdic acid by type B and C side chains and also by α,β unsaturated ketones. In spite of the fact that formaldehyde should be generated from two types of side chain (A and D) not active as the reducing substances as well as from the B and C types we regularly obtain much lower desoxycorticosterone equivalents on the basis of formaldehyde generation (cf. Tables 1, 2 and 3). A part of the excess measured by phosphomolybdic acid reduction may be accounted for by the non-alcoholic material in urine that acts on this reagent. On the basis of the data of 4 at most 20% of the neutral lipid of urine is non-alcoholic. We have recently had some evidence (Romanoff and Pincus 1949) that the neutral lipid fractions of human urine generate formaldehyde at a rate quite different from desoxycorticosterone. This may account for the lower equivalents obtained. Daughaday and collaborators (1948) give a brief report of formaldehydogenic steroid excreted by normal persons and find a range of 1.0 to 1.6 mgm. per day. This is much higher than the range we find (0.1 to 1.1 mgm. per day—see Tables 1 and 2), but they practice periodic acid oxidation on the total lipids, or urine whereas we have removed the alkali soluble lipids.

Again, the consistently lower desoxycorticosterone equivalents obtained by the copper reducing method (see Table 5) require some explanation. While the non-alcoholic reducing lipid may account for a part of the discrepancy there still remains other material measured by the phosphomolybdic acid reagent which is apparently not measured by the copper reagent. Such substances may in fact be present in the extraction mixture, but we are inclined to ascribe the difference to the fact, noted by Heard and Sobel (1946), that many steroids are extremely insoluble in the usual aqueous alkaline copper solution.

In view of the close correlation of the determinations made by the three methods we have employed, we believe that each tends to measure the same type of urinary corticoid, at least with the types of urines that we have analyzed. Any consideration, therefore, of the method of choice may properly be concerned with such questions as ease of handling, degree of variability and so on. The phosphomolybdic acid reduction method seems to us to be the most convenient since it avoids certain manipulations requisite in the other methods (e.g. periodic acid oxidation), it is more sensitive and can therefore be em-

ployed with smaller urine aliquots. It presumably will include a certain amount of non-specific material not measured by the other methods; but does not apparently exclude certain poorly soluble steroids that are excluded by the copper reduction method. As far as the variability of the measurements are concerned those made by the phosphomolybdic acid reduction method seem, if anything, to be less variable than those obtained by the other two methods. Thus the coefficients of variation of the data of Tables 2 and 5 are generally lower for the phosphomolybdic acid reducing data. A benzene:water partition of the neutral lipid appears to offer no special advantage since the ratio between the titers of the two incident fractions shows no special alteration with either diurnal stress or ACTH administration.

It should be deduced from the foregoing that there is no special utility in fractionating the neutral lipids or in employing a particular type of chemical assay to such fractions. The larger proportion of FL in the aqueous phase of the benzene:water partition, for example, indicates a means of segregating such substance if its special identification is desired. Further partitioning (e.g., extraction of the ketonic substances with Girard's reagent) would lead to presumably more specific information about special types of compound. For routine assays seeking data on the particular type of adrenocortical activity index given by the corticoids of urine the method of Heard and Sobel offers the advantages of economy, rapidity and relatively low variability.

SUMMARY

Aliquots of diurnal urine collections from 34 men and 34 women were analyzed for phosphomolybdic acid reducing substance (PRL) and for substance generating formaldehyde on periodic acid oxidation (FL) in the neutral lipid fractions. Both types of substances exhibited similar diurnal variations in output and significant correlations were obtained between both the absolute levels of output of each and the percentage increases of each occurring in the morning and rest of day collection.

The division of the data into two age groups reveals no significant difference with age in basal outputs of PRL or FL. Nor do the data demonstrate any consistent difference in the absolute level or rate of change of output of PRL and FL with age. The positive correlation of PRL and FL output holds for both age groups.

The partition of the neutral urinary lipids between benzene and water was performed with five diurnal specimens and with urine specimens collected by three normal, healthy men before and after the administration of adrenocorticotrophic hormone. On the average somewhat more phosphomolybdic acid reducing substance segregated into the benzene. The ratio of benzene:soluble to water:soluble activity did not appear to alter significantly in the various diurnal periods or

after ACTH administration. In the fractionated diurnal specimens the formaldehydogenic neutral lipids were determined and on the average the aqueous extracts contained more FL than the benzene extracts.

Non-alcoholic PRL was extractable from various types of urine specimen. It varied between 4% and 20% of the total PRL, and did not change notably in proportion after ACTH administration.

A comparison of PRL and cupric ion reducing lipid (CRL) in diurnal urines of 16 women and 2 men revealed similar excretion trends. Absolute output levels and percentage changes in output PRL and CRL correlated positively.

In terms of desoxycorticosterone equivalent outputs of FL are least, CRL intermediate, and PRL greatest. The bases for these differences are discussed.

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THE EFFECT OF ADMINISTERED ADRENAL CORTICAL HORMONES ON THE LIVER GLYCOGEN OF NORMAL AND SCORBUTIC GUINEA PIGS.¹

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CONSIDERABLE interest has developed concerning the role of cholesterol and ascorbic acid in the *in vivo* production of adrenal cortical hormones. This subject has been reviewed recently by Long (1947). Sayers, Sayers, Liang and Long (1946) have shown a rapid drop of the adrenal cholesterol and adrenal ascorbic acid in the rat and guinea pig with a simultaneous production of extra quantities of liver glycogen, when adrenocorticotrophic hormone is administered. These decreases of cholesterol and ascorbic acid appear to take place only in the adrenal glands. These and other workers, including Levin (1945), have shown adrenal cholesterol changes in various states of stress. The indication, but not conclusive proof, is that cholesterol and ascorbic acid of the adrenal gland are utilized, either directly or indirectly, in the production of cortical hormones. Vogt (1948) in her studies of adrenal secretions in dogs and cats could show no correlation between the contents of ascorbic acid and of cortical hormone in the plasma obtained from the adrenal bloods. Lowenstein and Zwemer (1946) reported the, as yet unconfirmed, isolation of an active cortical hormone containing the ascorbic acid moiety. These workers stated that their ascorbic acid containing compound was about equal to 11-dehydrocorticosterone in carbohydrate metabolic activity. Related to these observations is the report of Giroud, Santa and Martinet (1940) that scorbutic guinea pigs showed a decrease in the cortical hormone content of the adrenal gland. Banerjee (1943) has shown lower liver glycogen values for scorbutic guinea pigs than for normal animals, which he believes may be due to the lowered amount of cortical hormones available for liver glycogen production.

A further indication that ascorbic acid is involved in some un-

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known way in the glycogenic activity of cortical hormones was obtained by McKee, Cobbey and Geiman (1947) from liver glycogen studies with scorbutic guinea pigs. In this preliminary report it was stated that the ascorbic acid deficient guinea pigs show a sluggishness in their rates of gluconeogenesis and of glycogenolysis. Furthermore the administration of cortical hormones to the vitamin deficient guinea pigs does not stimulate liver glycogen production as it does in normal pigs.

This work resulted from malarial studies by McKee and Geiman (1946) attempting to determine the reasons for the prevention of malarial parasite growth in ascorbic acid deficient monkeys. Although the results to date do not give a solution of the malarial question they do indicate an interesting interrelationship between ascorbic acid and adrenal cortical hormone activity.

EXPERIMENTAL

The guinea pigs used were male animals weighing initially 225 to 275 grams. They were obtained by the University animal farm from a local animal breeder. All the animals were maintained during the course of the experiment on a diet of Purina rabbit chow, shown by analysis to be deficient in ascorbic acid. The normal non-scorbutic pigs were given a daily aseptic subcutaneous injection of 10 mg. of ascorbic acid. The ascorbic acid solution, which contained 40 mg. of Mallinckrodt U. S. P. material per cc. of double distilled water, was stabilized to oxidation by sterile filtration through an ultrafine (U. F.) Corning sintered glass filter and storing in the cold room at 5°C. The animals were given food and water *ad libitum* and the growth rates determined by weighing the pigs daily. The guinea pigs on the stock diet plus ascorbic acid showed a normal growth rate over the 21 to 30 day period, while the animals receiving no vitamin C supplement began losing weight in about 14 to 21 days. This loss of weight, which was indicative of impending ascorbic acid deficiency, was confirmed, by analysis of plasma, liver, kidneys, and adrenals for ascorbic acid according to the methods of Mindlin and Butler (1938) and Bessey (1948).

The scorbutic animals were usually employed for the experimental manipulations, of manual feeding followed by fasting and hormone therapy, when they had lost about half of their weight gain during the experimental period. The normal guinea pigs, which were started on the diet at the same time as the scorbutic animals, were utilized simultaneously for the experimental work. For the experimental procedure all of the guinea pigs, both normal and scorbutic, were fed manually two times, four hours apart, and zero time was taken as the time of the second feeding. The last ascorbic acid injection was given the normal animals at the time of the first manual feeding. The food given was a balanced synthetic ascorbic acid deficient diet (McKee and Geiman,

1946). A liquid mix was prepared by adding a measured amount of tap water to an equal weight of food, stirring and agitating to keep suspended. The animals were held gently and allowed to drink (not forced) 5 cc. of the mixture. The process was repeated 4 hours later with another 5 cc. of the food mixture (total of about 25 calories). All the animals took the food very well.

The cortical hormones, Parke-Davis Eschatin (50 dog units per cc.) and 11-dehydrocorticosterone in oil (5 mg. per cc.),³ were administered subcutaneously in divided doses. The Eschatin was given in 1.0, 0.5, 0.5, 0.5, and 0.5 cc. hourly doses and the animals sacrificed one hour after the last dose. The 11-dehydrocorticosterone was given in two 0.5 cc. doses 2 hours apart and the animals sacrificed 2 hours after the second dose. The hormone administrations were started so that the animals would be sacrificed at the end of the 24 hour period of fast.

The animals were sacrificed by a sharp blow on the back of the head and the peritoneal and pericardial cavities quickly opened. Blood was withdrawn from the heart by means of a 5 cc. syringe and #20 hypodermic needle and placed in a centrifuge tube containing heparin (0.5 mg.). The tissues (liver, kidneys and adrenals) were rapidly removed and a representative sample from each lobe of the liver (total of about one gm.) quickly weighed into an Erlenmeyer flask containing 5 cc. of 35 per cent potassium hydroxide. A rubber stopper carrying an air condenser was fitted into the mouth of the flask and the flask and contents placed in a boiling water bath. After about 10 minutes, when the tissue was completely dissolved, the flask and contents were cooled. One cc. aliquots of the liver-potassium hydroxide were used for the determination of liver glycogen according to the method of Good, Kramer, and Somogyi (1933). The glucose obtained by 2N sulfuric acid hydrolysis of the twice precipitated glycogen was determined by the method of Folin and Wu, according to Folin (1929).

Simultaneous with the preparation of the liver sample for glycogen analysis, the adrenals and samples of the kidneys, liver and spleen were weighed, ground in glass mortars with 3 per cent metaphosphoric acid and analyzed for ascorbic acid according to the method of Bessey (1938). The blood was centrifuged and the plasma analyzed for ascorbic acid according to the method of Mindlin and Butler (1938). Although the plasma of the scorbutic pigs consistently contained no ascorbic acid, the plasma from normal animals was extremely variable in ascorbic acid content (0.0 to 0.8 mg. per cent).

RESULTS

In order to determine the earliest time at which the liver glycogen of the animals could be reduced to a minimum by fasting, a series of normal and scorbutic animals were fasted 2, 12, 24, 36, 48 and 72

³ The 11-dehydrocorticosterone in oil was kindly furnished by Merck & Company through the courtesy of Dr. Augustus Gibson.

hours. The values for liver glycogen, for adrenal, liver, kidney and spleen ascorbic acid, and the ratios of adrenal weight to body weight are given in Table 1. An interesting point regarding these data is the almost complete loss of ascorbic acid from the adrenals of the scorbutic pigs contrasted to the smaller losses in the vitamin C content of the other organs of the vitamin C deficient animal, particularly the liver which still contains 20 to 25 per cent of the normal (non-fasted) amount. Even in the 72 hour fasting period, the loss of ascorbic acid from the adrenals is faster than from the other organs (adrenals

TABLE 1. CHANGES IN ADRENAL-BODY WEIGHT RATIOS, ORGAN ASCORBIC ACID AND LIVER GLYCOGEN FOR NORMAL AND SCORBUTIC GUINEA PIGS AFTER VARYING PERIODS OF FASTING

Fasting period hrs.	Adrenal wt.	Ascorbic acid				Liver glycogen
	Body wt. mg./gm.	Adrenals mg. %	Liver mg. %	Kidney mg. %	Spleen mg. %	gm. %
<i>Normal Guinea Pigs</i>						
2	0.34 (5)*	126.5 (5)	30.2 (5)	12.2 (5)	34.9 (5)	5.990 (5)
12	0.37 (8)	118.0 (8)	22.6 (8)	10.6 (8)	41.2 (8)	2.650 (7)
24	0.42 (17)	115.8 (17)	20.1 (17)	8.9 (17)	34.1 (17)	0.041 (17)
36	0.43 (8)	102.8 (8)	18.0 (8)	8.0 (8)	29.9 (8)	0.039 (8)
48	0.50 (9)	93.8 (9)	19.4 (9)	8.4 (9)	33.5 (9)	0.131 (9)
72	0.48 (7)	70.2 (7)	19.7 (7)	7.5 (7)	25.9 (7)	0.061 (7)
<i>Scorbutic Guinea Pigs</i>						
2	1.09 (5)	1.5 (5)	8.0 (5)	2.4 (5)	2.5 (5)	2.460 (5)
12	0.76 (6)	1.3 (6)	6.1 (6)	2.9 (6)	3.4 (6)	1.660 (6)
24	0.83 (16)	3.3 (17)	6.9 (17)	2.9 (14)	3.5 (17)	0.210 (17)
36	0.69 (5)	1.5 (8)	7.0 (8)	3.2 (8)	4.6 (8)	0.054 (8)
48	0.83 (5)	2.1 (6)	5.0 (6)	2.7 (6)	3.3 (6)	0.037 (6)
72	1.09 (10)	1.8 (10)	6.5 (4)	3.6 (4)	4.1 (4)	0.063 (8)

* The numbers in parentheses are the numbers of animals studied.

45, liver 35, kidneys 38 and spleen 26 per cent). Most important, however, is the absence in the scorbutic pigs of the normal rapid drop of liver glycogen to a minimal value in 24 hours and the subsequent (48 hour) rise. This normal compensatory increase in liver glycogen after 48 hours is due to the stimulation of the adrenals by the pituitary to produce gluconeogenic cortical hormones (Long, Katzin and Fry, 1940). Thus there appears to be in scorbutic guinea pigs a much slower than normal rate of glycogenolysis and gluconeogenesis, which is pictured graphically in Fig. 1.

In view of the work of Long, Katzin and Fry (1940), and of others showing the ability of adrenal hormones, particularly the 11 and 17 oxycorticoids, to produce liver glycogen in fasted animals, it seemed desirable to determine if the scorbutic guinea pigs had the ability to produce liver glycogen. As shown in Table 2 and Fig. 2 the Parke-Davis adrenal cortex extract, Eschatin, stimulated liver glycogen deposition. There was present 28 times more glycogen in the livers of the hormone treated animals than in the non-treated controls. Merck's 11-dehydrocorticosterone stimulated glycogen deposition with a pro-

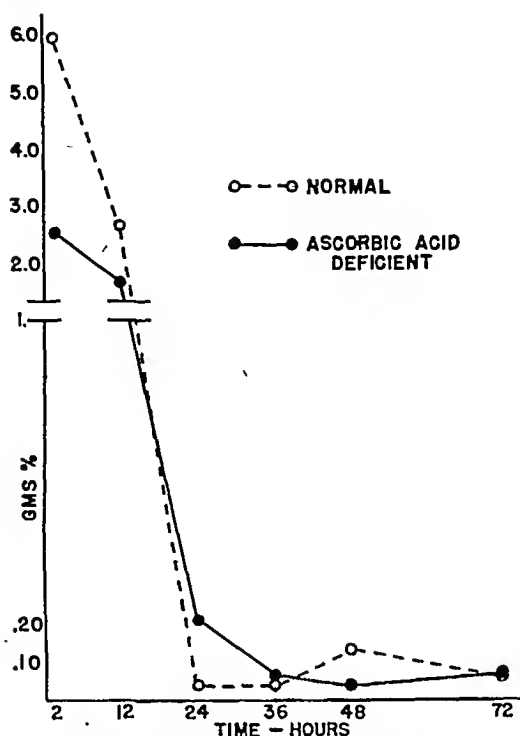


FIG. 1. Changes in liver glycogen during the fasting of normal and scorbutic guinea pigs.

duction of 8 times more glycogen than was present in the controls. Possibly the optimal effects were not obtained, particularly with the 11-dehydrocorticosterone in oil, due to inadequate time elapse following administration. The quantity of hormone available did not permit determining optimal conditions for administering the hormones.

TABLE 2. LIVER GLYCOGEN AND ORGAN ASCORBIC ACID AFTER INJECTION OF ADRENOCORTICAL HORMONES

Treatment	Adrenal wt.	Ascorbic acid				Liver Glycogen gm. %
	Body wt. mg./gm.	Adrenals mg. %	Liver mg. %	Kidney mg. %	Spleen mg. %	
<i>Normal Guinea Pigs</i>						
No treatment	0.42 (17)*	115.8 (17)	20.1 (17)	8.9 (17)	34.1 (17)	0.041 (17)
Eschatin (Parke-Davis)	0.42 (11)	120.9 (11)	24.5 (11)	11.7 (11)	48.3 (11)	1.160 (11)
11-dehydrocorticosterone (Merck)	0.38 (4)	95.7 (3)	18.4 (3)	10.3 (3)	38.7 (3)	0.329 (3)
<i>Ascorbic Acid Deficient Guinea Pigs (19-23 days)</i>						
No treatment	0.83 (16)	3.3 (17)	6.9 (17)	2.9 (14)	3.5 (17)	0.210 (17)
Eschatin (Parke-Davis)	0.94 (8)	1.5 (8)	4.6 (8)	1.8 (8)	2.5 (8)	0.065 (8)
11-dehydrocorticosterone (Merck)	0.86 (5)	2.7 (5)	6.1 (5)	1.1 (3)	3.2 (5)	0.117 (5)

* All animals were fasted 24 hours. The numbers in parentheses are the numbers of animals studied.

Contrasted to the striking deposition of liver glycogen in normal fasted animals, was the complete lack of deposition in scorbutic guinea pigs similarly treated with Eschatin and 11-dehydrocorticosterone (Table 2 and Fig. 2). In fact, there appeared to be even a slight, but possibly significant, hastening of the rate of glycogen removal from the livers of the scorbutic pigs.

Only one experiment with one group of animals has been carried out in an attempt to reverse the situation. In this experiment ascorbic acid (15 mg.) was administered during the period that the animals

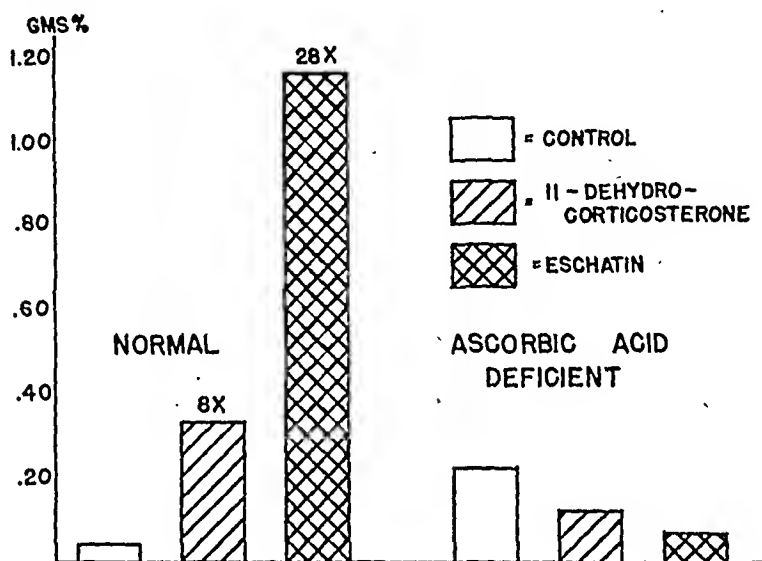


FIG. 2. The effects of Parke-Davis Eschatin and 11-dehydrocorticosterone on the liver glycogen of 24 hour fasted normal and scorbutic guinea pigs.

received the cortical hormones. No enhancement of liver glycogen deposition was obtained. Experiments are planned to determine when ascorbic acid must be administered to allow glycogen deposition with cortical hormone administration.

DISCUSSION

Under the conditions of our experiments the removal of ascorbic acid from the guinea pigs produced a condition incompatible with liver glycogen deposition when adrenal cortical hormones were administered. The reasons for this lack of cortical hormone activity are not known. Knowledge regarding the ease of reversibility with administered ascorbic acid of the above described condition in our scorbutic guinea pigs would be of value. Also the ability or inability of these animals to respond to injected adrenocorticotrophic hormone would be helpful in evaluating the situation. The complement of available cholesterol, the suspected building block for the cortical hormones

also must be kept in mind. Analyses for adrenal and blood cholesterol were not made in these experiments.

As stated by Sayers, Sayers, Liang and Long (1946), "The lack of resistance of the scorbutic guinea pigs to various stresses, together with certain similarities between scurvy and adrenal cortical insufficiency, is at least worthy of further study. . . ."

The work of Banerjee (1943; 1944a, 1944b) and of Banerjee and Ghosh (1946) is of interest in regard to the influence of ascorbic acid deficiency on carbohydrate metabolism. They have shown a degeneration of the β -cells of the pancreas with a marked diminution of the insulin content of the gland, a mildly diabetic picture, and significantly lowered liver glycogen. The work of Colowick, Cori and Slein (1947) indicates an interrelationship of insulin and certain adrenal cortical hormones, as shown by an inhibition with adrenal cortical extracts of the hexokinase system from the muscle of rats made diabetic by alloxan. This inhibition could be counteracted by the addition of insulin. Whether or not the administration of insulin would stimulate glycogen deposition in scorbutic guinea pigs has to be determined.

SUMMARY

The fasting of normal guinea pigs produces a rapid drop of liver glycogen which within 24 to 36 hours approaches base line levels. There is a subsequent rise in the liver glycogen within 48 hours due to the action of the pituitary on the adrenal glands with the consequent production of cortical hormones and liver glycogen deposition. The cortical hormones (Eschatin and 11-dehydrocorticosterone) stimulate glycogen production in the fasted animal. This situation is strikingly different in the fasted ascorbic acid deficient guinea pig. (1) The liver glycogen drops more slowly. (2) There is not, within 72 hours, a secondary rise in the liver glycogen which is observed in normal animals due to pituitary stimulation of the adrenals. (3) The cortical hormones tested have no stimulatory effect on the production of liver glycogen. It was also observed that the ascorbic acid content of the adrenals drops faster than the vitamin content of spleen, kidneys, and liver during both periods of fasting and during the time that the animals are on an ascorbic acid deficient diet.

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INDUCTION OF PSYCHIC ESTRUS IN THE HAMSTER WITH PROGESTERONE ADMINISTERED VIA THE LATERAL BRAIN VENTRICLE¹

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FOLLOWING estrone priming, the ovariectomized golden hamster responds to injections of progesterone in adequate doses by exhibiting psychic estrus as do also certain other common laboratory rodents, a fact first established by Frank and Fraps (1945) and subsequently verified by Kent and Liberman (1947) while studying vaginal smears associated with induced mating. While the threshold dose of progesterone necessary to effect psychic estrus when administered via a subcutaneous route has not been established, Frank and Fraps observed no mating among animals subjected to subcutaneous injection of 0.02 mg. of progesterone 24 to 48 hours after estrogen priming, but found that 0.05 mg. of progesterone was as effective in inducing mating as was 10 times this quantity. The purpose of the present paper is to report that doses of progesterone too small to effect psychic estrus when administered subcutaneously to ovariectomized, estrogen-primed females may be entirely adequate in inducing typical mating responses when introduced directly into the ventricles of the brain. In the present experiments no attempt was made to determine the minimum dose of progesterone necessary to effect psychic estrus by either the subcutaneous or the ventricular route. The possible significance of the findings will be discussed briefly.

MATERIALS AND METHODS

Animals utilized in the present experiment had been ovariectomized two weeks (in a few cases, slightly longer) before receiving the priming regimen of estrone. At the time of the initial estrone injection the vaginal smear was typical of castrate animals and none had exhibited estrous cycles or mating responses in the presence of males subsequent to ovariectomy. The regimen of estrone consisted of a series of injections of 50 μ g estrone daily for 6 days, a regimen sufficient to produce, on or before the sixth day, typical vaginal estrous smears devoid of leucocytes and composed of epithelial cells with polyhedral squamous cells predominating.

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Twenty-four hours after the last dose of estrone the animals were tested for mating responses. Of 63 animals employed to this point, one exhibited a mating response as a result of the effects of the estrone, and this animal was discarded. Both Frank and Fraps, and Kent and Liberman have shown that the effect of estrone in the induction of psychic estrus is erratic, and the exhibition of psychic estrus by this animal is not unexpected at the dosage level herein utilized. Two animals were subsequently lost in trephining.

Sixty animals were utilized in three groups as follows: Fifteen animals received subcutaneously 0.025 mg. progesterone (crystalline synthetic) in 0.05 cc. of olive oil previously neutralized with sodium bicarbonate and extracted with ether; 30 animals received 0.025 mg. progesterone in 0.05 cc. oil directly into the right lateral ventricle; 15 animals received 0.05 cc. oil alone, directly into the right lateral ventricle. Several additional animals were injected via the ventricle with oil containing India ink to determine the distribution of the granules and therefore the distribution of progesterone within the ventricular system. These latter animals were autopsied immediately after injection. The ink granules were found in the contralateral ventricle, in the hypothalamic region of the 3rd ventricle, and in the iter. It is presumed that, in the test animals, the progesterone underwent a similar dissemination.

Injection into the ventricle was via a trephine hole placed 6 hours earlier in the right parietal bone half way between the coronal and lambdoidal sutures and 1.5 mm. lateral to the sagittal suture. The animals were ambulatory and alert less than 10 minutes after trephining. A 26 gauge intradermal needle attached to a 1 cc. tuberculin syringe was directed ventrad and slightly laterad through the hole, the meninges, and the cerebral cortex while the animal was under light ether anesthesia.

The brains of the test animals receiving progesterone via the ventricles were fixed in ammoniacal alcohol after observations had been completed at the end of twenty-four hours and were sectioned transversely by hand. The pathway of the needle through the cerebral cortex and into the ventricle was observed in the sections with the aid of low power lenses. In most cases the needle penetrated the right lateral ventricle and the postero-dorsal-most extent of the right inferior horn. Trauma was probably not avoided entirely in the adjacent ventricular walls. In no instance had the needle been directed mediad, and as far as could be ascertained by gross examination the thalamus suffered no direct damage.

OBSERVATIONS

Of the 15 animals which received subcutaneously 0.025 mg. progesterone in oil all gave negative responses when subsequently tested for mating reactions. Testing commenced 20 minutes after the injection and a response was considered negative when the female failed to exhibit lordosis after 4 hourly exposures to a male of approximately 10 minutes each, followed by a single exposure 10 hours later. Antagonism on the part of the female necessitated the removal of the male before 10 minutes had elapsed in all except one instance. In previous experiments utilizing larger doses (0.1 mg.) of progesterone the present authors (1947) were able (after 3 hours) to elicit 33 positive responses in 33 trials. Frank and Fraps described the latent period of response to progesterone as not more than 2 or 3 hours.

Of the 15 animals receiving 0.05 cc. oil alone into the ventricle, all exhibited negative mating responses.

Of the 30 animals receiving 0.025 mg. progesterone in oil into the ventricle, all exhibited positive mating responses (lordosis and mating) at the end of 1 hour. Sixteen animals in this group were tested for mating responses 1 hour after injection and were positive at that time. One animal was tested at 50 minutes, 4 at 30 minutes, 2 at 25 minutes, 1 at 20 minutes, and 6 at 10 minutes, and all except one of the six in the last group were positive when tested. The animal which refused to exhibit lordosis 10 minutes after injection was positive when re-tested 5 minutes later. Lordosis was exhibited from 15 seconds (1 animal) to 2 minutes (7 animals) after introduction of a male into the cage with the test female for the first time after injection, most of them responding in 30 to 90 seconds. When tested at subsequent hourly intervals the reaction time to the male invariably decreased until, on the third hour, the female typically exhibited lordosis 10 seconds after exposure. The reflex following intraventricular injection was, in our experience, particularly strong, although no objective measurements were made. In one instance in which the male was withdrawn from the cage before having mounted a lordotic female the latter remained in rigid lordosis for 40 minutes.

Observation of vaginal smears 24 hours after the animals received 0.025 mg. progesterone via the ventricles revealed that these animals did not exhibit a vaginal response equivalent to that elicited in previous experiments (Kent and Liberman, 1947) in which the animals received, subcutaneously, a larger dose (0.1 mg.) of progesterone. The latter animals within 24 hours exhibited a condition reminiscent of typical diestrous smears of unoperated females, characterized by the presence of large numbers of leucocytes. In the present experiments utilizing smaller doses of progesterone leucocytes appeared in small numbers only and the smears apparently were tending toward an ultimate castrate condition. Jones and Astwood (1942) have shown in the rat that, if the dosage of progesterone is adequate, diestrus will follow an estrous smear even in the presence of excess estrogen.

Numerous investigations chiefly on the guinea pig have indicated a likelihood that one or more hypothalamic nuclei may be indispensable elements in the complex neural and hormonal integrating mechanism which ultimately brings about mating behavior at a specific stage of the estrous cycle in rodents (Bard, 1940; Brookhart *et al.*, 1940, 1941; Dey *et al.*, 1940, 1942; Dey, 1943). In the present experiment the following facts seem significant: 1. A dose of progesterone too small to effect psychic estrus when administered subcutaneously into the body wall will, if injected directly into the neurocoele of the forebrain of animals pretreated with estrone, facilitate the exhibition of a strong mating reflex. 2. The latent period of response to the progesterone is relatively short (10 minutes or longer). On the basis of these observations it is suggested that, in the intact animal, progesterone may act

postulated that the thyroid gland directly controls the gonadotrophic activity of the hypophysis in rabbits, hyperthyroidism inhibiting the production of follicle-stimulating hormone and inducing the elaboration of luteinizing hormone.

The technique of parabiosis has been used frequently in studying pituitary-gonadal interactions. Kallas (1930), Hertz and Meyer (1937), Bunster and Meyer (1938), and Biddulph, Meyer and Gumbreck (1940) have shown that when an ovariectomized female rat is placed in parabiosis with a normal female rat the ovaries of the latter undergo marked stimulation because of a gonadotrophic hypersecretion from the pituitary gland of the ovariectomized parabiont. The present parabiosis experiments were undertaken to study the effect of thyroxine on this pituitary-ovary interrelationship in an attempt to determine the nature of its action on the reproductive system.

METHODS AND PROCEDURE

A total of 157 pairs of litter-mate female albino rats were joined in parabiosis according to the method of Bunster and Meyer (1933) except that metal skin clips were used instead of silk sutures.² The operations were performed between the 31st and 33rd days of life upon animals weighing in excess of 70 grams. In all cases the right hand partners were ovariectomized at the time of parabiotic union. The ovaries of the left hand partners remained intact to serve as a measure of the pituitary gonadotrophic effectiveness of the ovariectomized parabionts. Twenty-three of the pairs were reserved as uninjected controls. The remaining 134 pairs were injected once daily for periods of 10 days with dosages of thyroxine³ ranging from 0.025 mg. to 0.15 mg. In 68 of the experimental pairs the ovariectomized right hand partners were injected and in 66 pairs the intact left hand partners received the injections. Both experimental and control pairs were autopsied on the 11th day after the operations at which time the ovaries of the normal partners were freed of extraneous tissue and weighed to the nearest milligram. The qualitative response of the ovaries, the degree of uterine stimulation, and the presence or absence of vaginal canalization were also noted.

The pituitary glands of most of the thyroxine-treated and control pairs were pressed out individually between microscope slides and stored in a desiccator. After periods of storage not exceeding 6 months, the dried pituitary tissue was powdered and weighed out in 2.5 mg. quantities. Usually from 3 to 5 desiccated glands were required to make up this weight of powder. The glands of intact partners were always prepared separately from those of ovariectomized partners and glands of intact partners with unstimulated ovaries were never pooled with those of intact partners showing ovarian stimulation. Each weighed unit of powder was suspended in 5 cc. physiological saline and thoroughly shaken. The glands were assayed in 21 day old female mice, each mouse receiving 0.5 cc. of the suspension twice daily for periods of 4½ days. The pituitary powder from a given group of ovariectomized partners was assayed simultaneously in litter-mate mice with that

² The data were obtained during the years 1939-1941.

³ Roche-Organon.

obtained from their intact parabiotic mates. In most cases a third litter mate mouse served as an uninjected control animal. All mice were autopsied on the morning of the 6th day after the beginning of the injections. The ovaries and the uteri were weighed to the nearest tenth of a milligram. Assays were also performed on pituitary glands from 41-43 day old ovariectomized and normal single female rats which had received no thyroxine.

Immediately before autopsy, several of the thyroxine-treated and control pairs were placed under ether anesthesia and bled from the heart, the blood from ovariectomized and normal partners being collected separately. The blood was centrifuged and the serum obtained was stored in a frozen condition. The serum was assayed in 21 day old female mice, each animal receiving 0.5 cc. 4 times daily for $4\frac{1}{2}$ days. Thus, each mouse received a total dosage of 9 cc. serum. As in the pituitary assays, serum from ovariectomized and normal donors was injected separately in litter-mate mice. Autopsies were performed on the morning of the 6th day after the beginning of the injections. Ovarian and uterine weights were recorded and the condition of the vaginas was noted.

RESULTS AND DISCUSSION

The toxic effect of thyroxine was quite marked, particularly in those animals receiving the highest daily dose. Thirty-three of the 134 experimental pairs died before the end of the injection period. Data from these animals are not included in the following discussion. Among the surviving thyroxine-treated pairs there was usually a definite body weight loss, although there were several exceptions and the majority of animals appeared to be in good health. There were no deaths among the control pairs and most of them gained a few grams in weight during the period of parabiosis.

The data in Table 1 show that in approximately 68% of the surviving experimental pairs, thyroxine prevented ovarian hypertrophy in the intact partners. In these animals the ovaries weighed less than 20 mg., being no heavier than those of normal single rats of the same age. Qualitatively, the ovaries contained only small follicles. The uteri in all cases were unstimulated and the vaginas of both partners were closed. In the control group, only 2 animals (9%) showed a negative ovarian response. The intact partners of the remaining untreated pairs exhibited definite ovarian and uterine stimulation. Statistically, the difference in ovarian weight averages (26 mg. for the thyroxine-treated pairs and 80 mg. for the control pairs) is highly significant.

An attempt was made to obtain a quantitative measure of the effectiveness of thyroxine in preventing ovarian hypertrophy by injecting it at 4 dose levels. It will be noted (Table 1) that the lowest daily dose of 0.025 mg. was least efficient in this respect. When the amount was raised to 0.05 mg. per day a much higher incidence of ovarian inhibition occurred. Doses of 0.10 mg. and 0.15 mg. were also effective in preventing ovarian hypertrophy but were not significantly more so than the 0.05 mg. dose. Amounts greater than 0.15 mg. were

TABLE. 1 THE INCIDENCE OF OVARIAN INHIBITION AND OVARIAN WEIGHT AVERAGES OBTAINED IN THE INTACT PARTNERS OF THYROXINE-TREATED AND CONTROL PARABIOTIC RATS

Partner injected	Daily thyroxine dose (mg.)	Numbers of pairs	Pairs with unstimulated ovaries	Average ovarian weights (mg.)
Intact	0.025	9	2	55 ± 11.2*
	0.05	9	8	32 ± 19.6
	0.10	23	16	21 ± 4.4
	0.15	8	6	21 ± 5.7
Totals intact partners injected		49	32	29 ± 8.7
Ovariectomized	0.05	10	2	43 ± 10.1
	0.05	19	16	18 ± 3.5
	0.10	15	11	27 ± 9.3
	0.15	8	8	12 ± 1.2
Totals ovariectomized partners injected		52	37	25 ± 6.5
Totals thyroxine-treated pairs		101	69	26 ± 7.3
Control pairs		23	2	80 ± 9.1

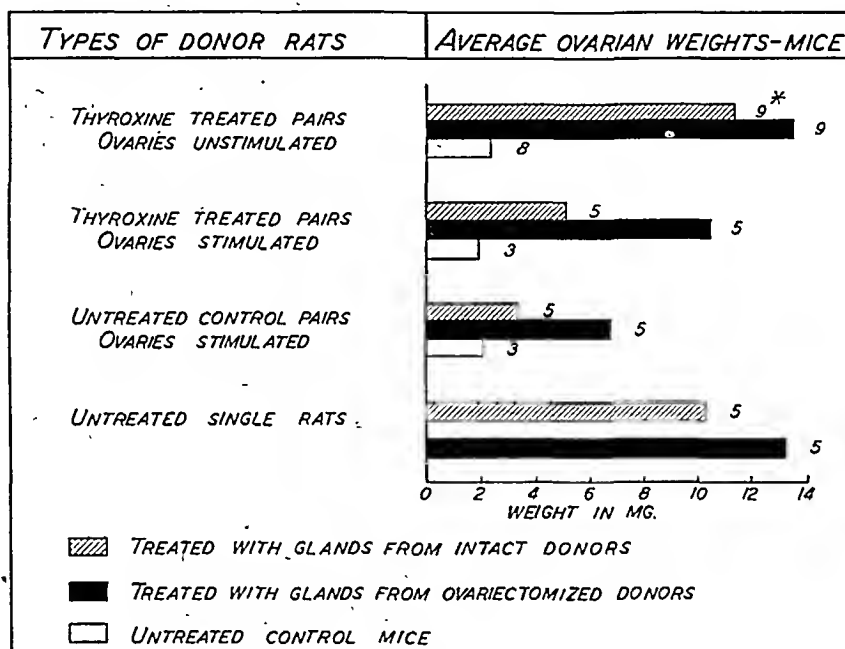
* Standard error.

not administered because of the toxic effect of thyroxine. Thus, total ovarian inhibition was not obtained with any of the 3 highest doses. Small numbers of pairs at each level were, for no obvious reason, comparatively resistant to thyroxine and showed varying degrees of ovarian and uterine stimulation. The higher resistance of these animals is also indicated by their relatively slight body weight loss. This averaged 7 gm. per pair in the animals with stimulated ovaries as compared to 15 gm. per pair in the rats showing no ovarian stimulation. Meyer and Hertz (1937) and Biddulph, Meyer and Gumbreck (1940) produced complete ovarian inhibition in intact parabionts by administering estrone, estradiol, and estriol to the ovariectomized partners. Thyroxine, therefore, was not as effective as estrogens in preventing ovarian hypertrophy.

Administration of thyroxine to the intact parabionts produced a negative ovarian response in 65% of the animals so treated. When the hormone was administered to the ovariectomized partners, 71% of their normal parabiotic mates showed unstimulated ovaries. The similarity of these results indicates that thyroxine passed freely across the parabiotic union, inducing a hyperthyroid condition in both partners. As a result, the inhibitory effect of thyroxine on the ovaries of the intact parabionts was equally manifest regardless of which partner was injected. In this respect, the action of thyroxine differs from that of the sex hormones. Biddulph, Meyer and Gumbreck (1941) and Shipley, Meyer and Biddulph (1943) have shown that many times the minimum stimulating doses of estrogens and androgens must be

administered to one parabiont before evidence of transfer appears in the other.

The parabiosis data demonstrate that thyroxine was effective in preventing ovarian hypertrophy in the intact parabionts, but they provide no direct evidence as to the mechanism of this action. Under the conditions of the experiment, thyroxine may have: (1) inhibited the pituitary glands by preventing the production or release of gonadotrophic hormone; or (2), rendered the ovaries of the intact parabionts refractory to gonadotrophic stimulation. In order to study



* Number of mice in assay.

FIG. 1. Average ovarian weights of immature mice injected with 2.5 mg. suspensions of dried pituitary powder from thyroxine-treated and control parabiotic rats and from untreated ovariectomized and intact single rats.

these possibilities, assays were performed on the pituitary glands of thyroxine-treated and control pairs.

Average ovarian weights of mice receiving 2.5 mg. amounts of dried pituitary tissue from various groups of intact and ovariectomized parabionts are represented graphically in Figure 1. Also shown are the results of assays of glands from ovariectomized and normal single rats.

Assay data from normal donors show that pituitary glands from the intact partners of thyroxine-treated pairs with unstimulated ovaries produced marked ovarian hypertrophy in mice. In this respect they were of about the same degree of potency as glands from untreated

single rats of the same age having immature ovaries. However, they were more than twice as effective in the mouse assays as pituitaries from the intact partners of thyroxine-treated pairs with stimulated ovaries and more than 3 times as potent as glands from the corresponding partners of untreated control pairs in which the ovaries had also undergone marked hypertrophy.

It is evident from these data that thyroxine treatment did not prevent the production of gonadotrophic hormone by the pituitary glands of the intact parabionts. Instead, the factor controlling pituitary gonadotrophic potency in these animals appeared to be the functional condition of their ovaries; or, in other words, the blood level of estrogens. Thus, in both experimental and control parabionts with enlarged ovaries the pituitary glands were low in gonadotrophic content. In other parabiosis experiments, Meyer, Biddulph, and Finerty (1946) have also provided evidence that the production of gonadotrophin by the pituitary glands of intact parabionts is inhibited by estrogens from their own stimulated ovaries. In most hyperthyroid pairs, on the other hand, unstimulated ovaries in the intact partners were associated with relatively potent pituitary glands. A similar condition existed in immature single rats. These data suggest that thyroxine decreases the sensitivity of the ovaries to gonadotrophin and as a result the blood estrogen level is low. This favors production and perhaps storage of gonadotrophin by the pituitary gland.

In the ovariectomized donors the same relative order of pituitary gonadotrophic potencies prevailed as in the intact donors. Thus, glands from the ovariectomized partners of thyroxine-treated pairs with unstimulated ovaries and hypophyses from ovariectomized single rats produced the greatest ovarian response in mice. They were only slightly more potent than glands from thyroxine-treated pairs with stimulated ovaries, but were twice as effective as pituitaries from ovariectomized control parabionts. Here again, there was no evidence of a decreased production of gonadotrophic hormone as a result of thyroxine treatment. In most hyperthyroid pairs, relatively potent pituitary glands in the ovariectomized partners were associated with unstimulated ovaries in the normal parabiotic mates. The reverse was true in control pairs and in those experimental pairs which were relatively resistant to thyroxine.

A possible interpretation of these data is based on the concept of utilization of gonadotrophin by the ovaries as suggested by the experiments of Seidlin (1940) and Heller, Heller, and Sevringhaus (1942). It can be postulated that the ovaries of the intact partners of control pairs were comparatively sensitive to gonadotrophic stimulation. Consequently, they became enlarged by removing and metabolizing gonadotrophin from the blood stream. This resulted in an increased output of hormone from the pituitaries of the ovariectomized partners and a lowering of their gonadotrophic content. Supporting this con-

cept are the triple parabiosis experiments of Plagge (1948) which indicate that the gonadotrophic secretion from the castrated left hand partner was so completely utilized by the enlarged ovaries of the middle partner that ovarian hypertrophy did not occur in the right hand member of the trio. In most hyperthyroid pairs it can be supposed that the ovaries of the intact partners were insensitive to stimulation. Hence there was little utilization of gonadotrophin from the hypophyses of the ovariectomized parabionts. This resulted in a storage of hormone in the glands of those animals and a higher potency at assay. In this respect, they resembled the glands of ovariectomized single rats.

The results of the pituitary assays pointed to a direct inhibitory effect of thyroxine on the ovaries. However, the possibility remained that thyroxine had prevented the release of gonadotrophin from the

TABLE 2. INDIVIDUAL AND AVERAGE OVARIAN AND UTERINE WEIGHTS OF MICE INJECTED WITH BLOOD SERUM FROM THYROXINE-TREATED AND CONTROL PARABIOTIC RATS

Types of Donor Rats	Recipient mice								
	Serum from Ovariectomized partners			Serum from Intact partners			Untreated control Mice		
	Ov. Wt. (Mg.)	Ut. Wt. (Mg.)	Vag.	Ov. Wt.	Ut. Wt.	Vag.	Ov. Wt.	Ut. Wt.	Vag.
Thyroxine-treated pairs:	2.2	16.0	Open	2.2	15.7	Closed	2.4	10.8	Closed
Ovaries of intact	3.1	18.0	Open	3.0	14.0	Open	2.8	15.0	Closed
partners unstimulated	3.1	22.3	Open	2.1	12.4	Closed	1.6	13.5	Closed
Averages hyperthyroid serum	2.8	18.8		2.4	14.0	—	2.3	13.1	—
Control pairs:	2.0	65.3	Open	1.2	12.7	Closed	—	—	—
	1.4	20.0	Open	1.8	13.7	Closed	1.9	13.6	Closed
Ovaries of intact	4.3	13.8	Closed	3.0	12.9	Closed	3.3	12.5	Closed
	4.8	15.4	Closed	3.6	11.7	Closed	3.3	12.5	Closed
partners stimulated	2.1	21.4	Open	1.8	15.9	Open	1.5	16.0	Closed
Averages control serum	2.9	27.2		2.3	13.4		2.2	13.7	

pituitary glands, thereby lowering its concentration in the blood stream. To test this assumption, serum assays were performed.

Table 2 shows that serum from the ovariectomized partners of both hyperthyroid and control pairs produced no definite ovarian hypertrophy in mice. However, some uterine stimulation occurred, and in 6 of the 8 animals receiving this treatment the vaginas were open at autopsy. Assays of serum from intact parabionts of both experimental and control pairs were negative with the exception of one mouse in each group in which the vagina was open. The slightly greater serum potency of ovariectomized partners may have been the result of an accumulation of gonadotrophin in the blood of those animals caused by a retarded passage across the parabiotic union.

The serum assays, although limited, show no significant difference in blood gonadotrophic content between experimental and control

pairs. The somewhat higher uterine weight average obtained in mice treated with ovariectomized control serum was due to one animal in which the uterus had undergone marked hypertrophy. These data are in accord with the more extensive assays of Smelser (1939) who found no difference in gonadotrophic effectiveness between hyperthyroid and control serum from castrated rats. It would appear, therefore, that thyroxine administration did not change the rate of pituitary gonadotrophic secretion.

In conclusion, it can be stated that the female reproductive system in parabiotic rats was affected by thyroxine in a manner similar to that reported in the literature for single rats. The most striking manifestations were ovarian inhibition in the intact parabionts accompanied by an increased pituitary gonadotrophic potency in both partners. This increased potency in the intact partners appeared to be caused by the low blood estrogen levels resulting from hypofunctional ovaries. Evidence is provided that the higher potency of glands from ovariectomized hyperthyroid parabionts was due to a lack of utilization of their secretions by the ovaries of the intact partners. The serum assays indicate that the unstimulated ovaries of experimental pairs were under the influence of about the same blood concentrations of gonadotrophin as were the stimulated ovaries of control pairs. This situation is comparable to that observed in the previously cited experiments of Fluhman (1934) and others, in which administration of thyroid substances to single rats reduced the effectiveness of injected gonadotrophins in stimulating the ovaries. Thyroxine, therefore, probably produces ovarian inhibition in the rat by directly decreasing the sensitivity of the ovary to gonadotrophic stimulation.

SUMMARY

Administration of thyroxine to ovariectomized female-intact female parabiotic rats prevented ovarian hypertrophy in 68% of the intact partners. This effect occurred regardless of which partner was injected.

Pituitary glands from both the intact and ovariectomized partners of thyroxine-treated pairs with unstimulated ovaries had greater gonadotrophic activity than glands from the corresponding partners of either hyperthyroid or control pairs with stimulated ovaries.

Hyperthyroid blood serum contained the same amount of gonadotrophic hormone as control serum. However, serum from the ovariectomized partners of both groups was slightly more potent than serum from the intact partners.

Thyroxine appears to exert its inhibitory effect on the female reproductive system by decreasing the sensitivity of the ovary to gonadotrophic hormone.

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HISTOLOGICAL, CYTOCHEMICAL AND PHYSIOLOGICAL OBSERVATIONS ON THE REGENERATION OF THE RAT'S ADRENAL GLAND FOLLOWING ENUCLEATION¹

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WITH THE accumulation of evidence for a functional zonation of the adrenal cortex, as postulated by Swann (1940) and supported by Sarason (1943) and by Deane and Greep (1946), the origin and functional capacity of new cortical tissue arising after enucleation of the gland acquire new significance.

Adrenal enucleation involves slicing open the capsule and extruding the medulla and most of the cortex (Ingle and Higgins, 1938). If both glands are so enucleated, or if one gland is enucleated and the other totally removed, regeneration of cortical tissue takes place rapidly so that the original volume is restored in about a month. (Because of the regeneration of the cortex, the operation is generally termed demedullation.) The regeneration is dependent upon the presence of the pituitary.

The origin of the new parenchymal tissue is still a matter of controversy. Originally Ingle and Higgins described regeneration as taking place from the capsule, but Higgins (personal communication²) has informed us that they meant the capsular region, i.e., the capsule and the subcapsular cortical cells. Because of the reports of Zwemer, Wotton and Norkus (1938), Baker and Baillif (1939), Gruenwald and Konikov (1944) and Baxter (1946); however, it is often assumed that the origin of new cortical cells is principally from fibroblasts in the capsule. In addition to this problem of the involvement of the cap-

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² Statement from Dr. G. M. Higgins, August, 1948: "In our studies, a number of years ago, upon the regeneration of the adrenal gland Dr. Ingle and I undertook a study of cortical regeneration in adrenal glands of rats from which the medulla and most of the cortex had been expressed. It is obvious that such procedures can not entirely free the capsule of all of the adjacent cortical tissues, so that small islands of glomerulosa cells always remained adherent to the capsule. Whereas we used the term capsule to define that portion of the adrenal which remained in place upon enucleation, it is certain that the number of glomerulosa cells which persisted formed foci from which, at least very largely, subsequent regeneration of cortical tissue occurred."

sule, the question further arises as to whether or not peripheral tissue forms a cortex capable of secreting hormones which influence both salt and sugar metabolism. This is of especial importance because evidence is accumulating to show that the zona glomerulosa secretes those hormones which influence salt and water metabolism (Deane, Shaw and Greep, 1948), whereas the zona fasciculata secretes those which affect protein and carbohydrate metabolism (Bergner and Deane, 1948). The present study was undertaken to determine, in so far as possible, both the origin of the new tissue and its functional characteristics. Histological, cytochemical and physiological methods have been employed.

MATERIAL AND METHODS

The animals used in these experiments were adult (200 gm.) male rats of the Long-Evans strain. Bilateral adrenal enucleations were performed under ether anesthesia. Each adrenal gland was exposed and its capsule sliced open at one pole. With small curved forceps slight pressure was exerted at the opposite pole, causing the extrusion of a solid core, which was removed. The capsule was then "milked" between closed forceps to remove all parenchymal tissue possible. The capsule of the enucleated gland was restored to position in order to permit regeneration. Three types of experiments were performed.

1) *Histology and cytochemistry.* Groups of 3 rats were killed 1, 3, 8, 18 and 32 days after operation.

One adrenal from each group of 3 rats was fixed in Bouin's fluid, embedded in paraffin, and sectioned serially at 10 μ . The sections were stained with Harris' hematoxylin and eosin.

One adrenal from each rat was fixed for 48 hours in 10 per cent neutralized formalin, then washed in running tap water for an hour and sectioned on the freezing microtome at 15 μ . One section was stained with sudan IV and Harris' hematoxylin, one with sudan black B, and one by the Schiff "plasma" method; these were mounted in glycerin jelly. Two other sections were mounted unstained in glycerin, one of which was untreated and the other extracted for one-half hour in acetone at room temperature. These two sections were compared under polarizing and fluorescence microscopes for acetone-soluble droplets showing birefringence and a greenish-white autofluorescence. The group of reactions employed characterizes the lipid droplets of steroid-secreting glands, and variations in reactivity have been correlated with different degrees of functional activity (Dempsey and Wislocki, 1946; Dempsey, 1948).

Another adrenal from each group of 3 rats was fixed in an alcoholic-acid silver nitrate solution for the demonstration of ascorbic acid (Barnett and Bourne, 1940). After fixation for one-half hour it was transferred to an acidified hypo solution for an hour (Deane and Morse, 1948). Then it was washed in running water, embedded in paraffin, and sectioned at 5 μ . The nuclei were stained with pararosaniline.

2) *Sugar metabolism.* Blood-sugar levels were recorded during a period of complete starvation in 5 normal rats, in 10 rats whose adrenals had been enucleated for 1 month, and in 5 rats that had been adrenalectomized 1 week

previously and supplied with a 1 per cent sodium chloride solution for drinking water. Blood samples were taken from the tail by snipping off the tip. Samples were collected at 8 A.M. at the beginning of the fast and thereafter 12, 24, 36, 48, 72, 96 and 120 hours later. During the period of starvation the animals were kept in cages with wire mesh bottoms and were supplied with tap water, or 1 per cent sodium chloride solution in the case of the adrenalectomized rats, *ad libitum*. The adrenalectomized rats succumbed in 3 to 4 days, the others were still alive at the end of 5 days.

The level of sugar in the blood was determined by the modification of Folin's micro-method given in the Rubicon Co. manual for the Evelyn Photoelectric Colorimeter. The readings were made on an Evelyn Colorimeter.

3) *Injection of desoxycorticosterone acetate*. Four rats which had their adrenals enucleated for $1\frac{1}{2}$ months received daily injections for a month of 2 mg. desoxycorticosterone acetate (Percorten, Ciba³) in oil. As controls, 2 untreated rats with demedullated glands were killed at the beginning and 2 at the end of the injection period. The adrenals were fixed in formalin and stained by the group of methods for indicating the presence of ketosteroids given above.

OBSERVATIONS

Histology and cytochemistry of the regenerating adrenal cortex

1 day. Twenty-four hours after enucleation, the adrenal gland appeared of normal size and shape both grossly and in section. The capsule was somewhat edematous. Beneath a large portion of the capsule no cortical cells could be found (Figs. 2 and 7), but in some places a few rows of cells remained, sometimes as many as 6 or 7 (Figs. 1 and 7). Only occasional abnormal mitotic figures in cortical

³ The Percorten was generously supplied by the Ciba Pharmaceutical Products, Inc., Summit, N. J.

PLATE 1. Description of Figures.

All photomicrographs on this plate are from enucleated adrenal glands which were fixed in Bouin's fluid, embedded in paraffin, sectioned serially at $10\ \mu$, and stained with hematoxylin and eosin. Photographed with green and yellow filters combined.

FIG. 1. Portion of adrenal enucleated for 1 day. At this site, about 5 rows of cortical cells remain attached to the capsule. The central part of the gland is filled with a blood clot and considerable accumulations of polymorphonuclear leucocytes. $\times 200$.

FIG. 2. Another portion of same adrenal gland shown in Fig. 1. Here no parenchymal cells remain attached to the capsule. Compare with Fig. 7. $\times 200$.

FIG. 3. Portion of adrenal gland enucleated for 3 days. The capsule appears edematous and its cells enlarged. The cortical cells now occur all around the gland and have formed short cords. M, mitotic figure in parenchymal cell. The central clot has become fibrous. $\times 200$.

FIG. 4. Portion of adrenal gland enucleated for 8 days. The capsule still appears edematous. The cords of cells have lengthened somewhat. Mitotic figures (M) and recently divided cells (D) occur throughout. Connective tissue is developing in the center. $\times 200$.

FIG. 5. Portion of adrenal gland enucleated for 18 days. The capsule has returned to essentially normal appearance. The cords have elongated considerably but often form whorls (compare Fig. 10). Mitoses (M) and recently divided cells (D) prevalent. $\times 200$.

FIG. 6. Portion of adrenal gland enucleated for 32 days. The gland now possesses its normal architecture—narrow outer zona glomerulosa, a broad middle zona fasciculata, and an internal zona reticularis. No mitotic figures were found in this gland. $\times 160$.

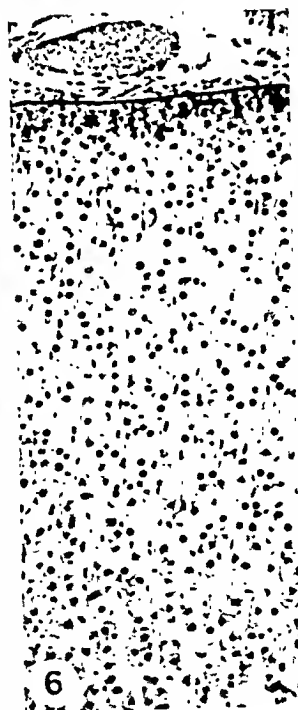
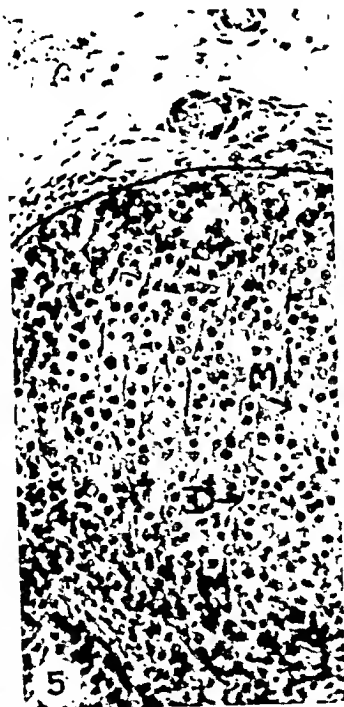
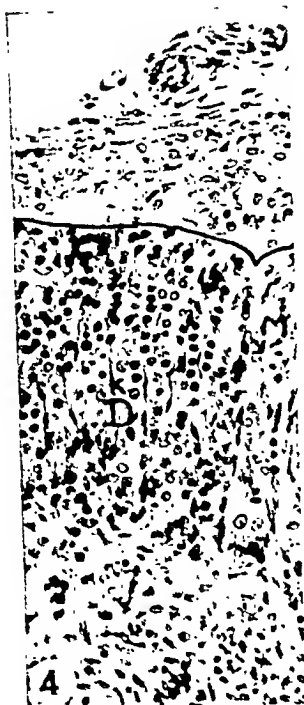
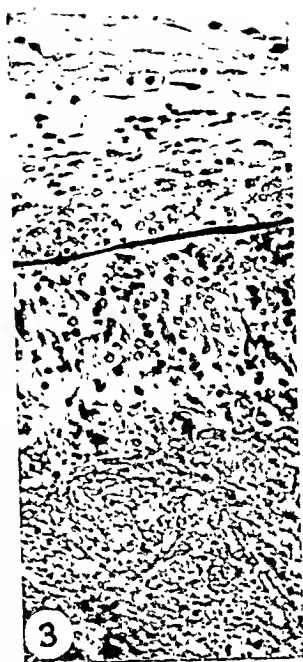
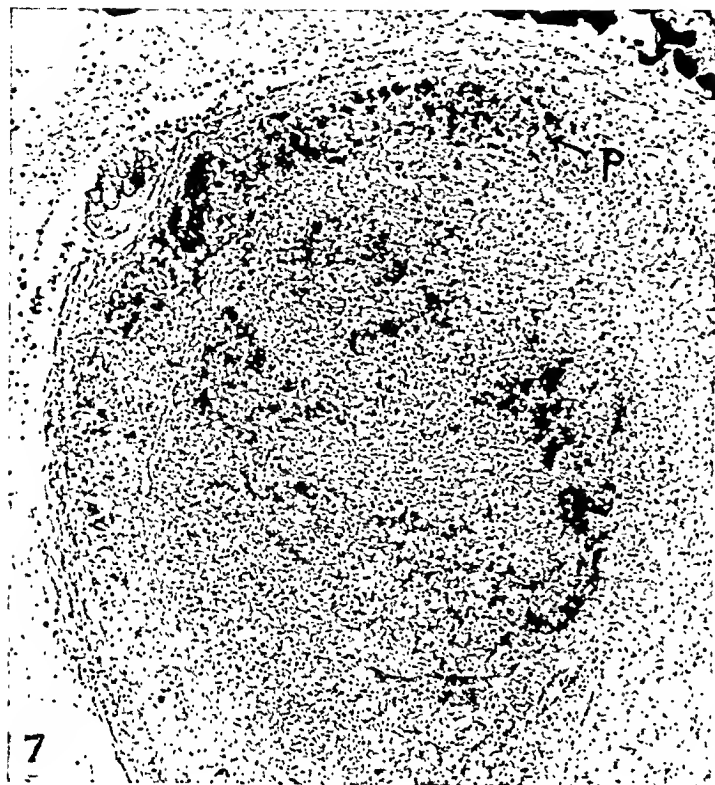


PLATE 1



cells were observed at this time (Deane and Morse, 1948, Fig 9). The central area was filled with a blood clot containing cellular debris, hemosiderin and numerous polymorphonuclear leucocytes. No loose parenchymal cells could be identified with certainty in the central clot.

One day after enucleation the few remaining parenchymal cells all contained a few sudanophilic droplets (Fig. 7) which were also Schiff-positive, autofluorescent and birefringent. The droplets were large and the birefringent crystals coarse. The cells lacked granules of reduced silver (Deane and Morse, Fig. 9).

3 days. Three days after enucleation the central clot was partially resorbed, causing the gland to appear collapsed and the capsule scalloped (Fig. 8). The capsule appeared more edematous than before and its cells, particularly those in its inner layer, were swollen and vacuolated (Figs. 3 and 8). No mitotic figures were found in capsular cells. The blood vessels in the capsule were distended with blood, suggesting that circulation had been restored. Within the central clot, leucocytes appeared degenerate and vacuolated (Fig. 8), and connective-tissue cells were making their appearance.

Cortical strands 8 to 12 cells in length extended down from the capsule all around the section. The cytoplasm of these cells varied in stainability—some cells stained with eosin, some failed to stain. Occasional mitotic figures were found, generally at about the midpoint of the cell columns (Fig. 3).

Only some of the parenchymal cells contained lipid droplets at 3 days (Fig. 8); these droplets exhibited the other lipid reactions employed as well, the birefringence being coarse. No ascorbic acid occurred in any cortical cells. Macrophages and degenerating leucocytes in the central area also contained sudanophilic droplets, but these

PLATE 2

All photographs on this plate are of enucleated adrenal glands which were fixed in 10 per cent neutralized formalin for over 48 hours, washed, and sectioned on the freezing microtome at 15 μ . X90.

FIG. 7. Adrenal gland enucleated for 1 day. Sudan IV and hematoxylin. Under the capsule fatty cortical cells (P) occur at some points, are absent elsewhere (compare Figs. 1 and 2). The dark material in the central clot is not fat but blood pigment. Peri-adrenal fat above.

FIG. 8. Segment of adrenal gland enucleated for 3 days. Sudan IV and hematoxylin. The gland has collapsed somewhat and the surface appears scalloped. The cortical cells (P) contain little lipid material. Macrophages and masses of degenerating blood cells in the internal scar contain considerable fat.

FIG. 9. Segment of adrenal gland enucleated for 8 days. Sudan IV and hematoxylin. Whorl of cortical cells above, mass of degenerating white blood cells below. Of the cortical cells, only the outermost ones contain any lipid.

FIG. 10. Segment of adrenal gland enucleated for 18 days. Schiff "plasmal" reaction. The cortical tissue occurs in whorls around the central scar. The cortical cells all contain droplets exhibiting the Schiff reaction.

FIG. 11. Segment of adrenal gland enucleated for 32 days. Sudan IV and hematoxylin. The parenchyma has regenerated to approximately normal dimensions and the cells all contain fatty droplets. Periadrenal fat above.

in the 3 groups. The rats with demedullated adrenals behaved similarly to the intact animals. The level of blood sugar dropped for 36 to 48 hours, then rose to the original level. The adrenalectomized animals began with a somewhat lower blood sugar, and the level fell steadily until coma and death ensued at 3 to 4 days.

Injection of desoxycorticosterone acetate

Since the structure and cytochemical appearance of the adrenal cortex were restored to normal a month after enucleation, it was of

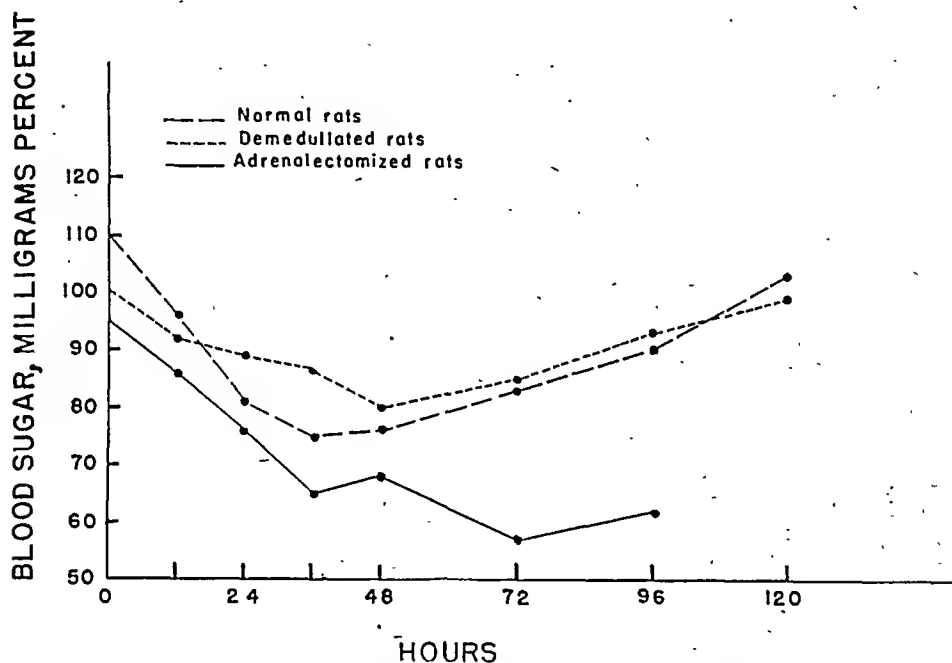


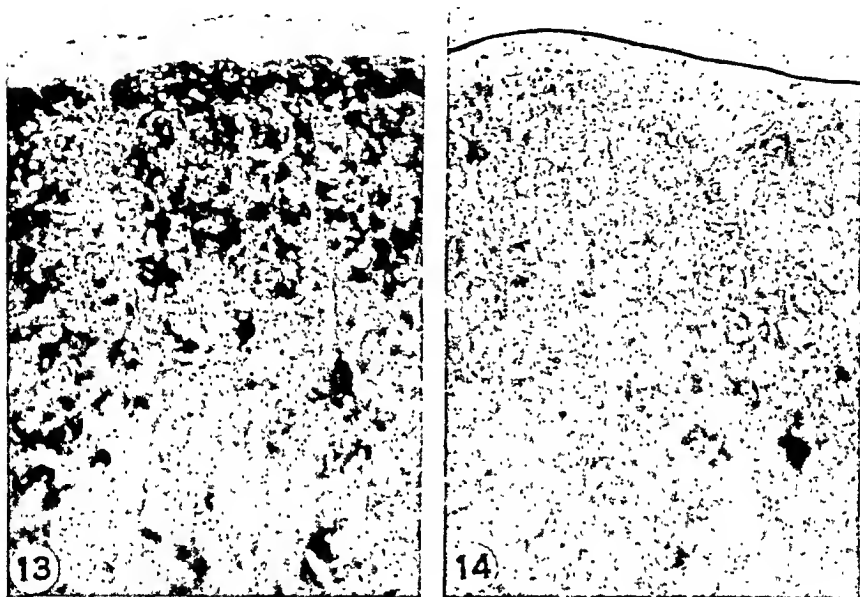
FIG. 12. Changes in blood-sugar levels in groups of normal rats, rats with regenerated adrenal glands, and adrenalectomized rats during a period of complete starvation.

interest to learn if the normal functional zonation was restored as well. In normal animals the injection of desoxycorticosterone acetate appears to cause a suppression of secretory activity in the zona glomerulosa (Greep and Deane, 1947), a finding which suggests that the salt-retaining hormones are secreted by this zone.

Four rats whose adrenals had regenerated for $1\frac{1}{2}$ months were injected daily for a month with 2 mg. desoxycorticosterone acetate, then killed. Their adrenals were compared with the adrenals of 2 animals with demedullated glands killed at the beginning of the injection period and 2 others killed at the end.

The regenerated adrenals from animals without hormone treatment presented the normal appearance, with a large amount of lipid material displaying the various lipid reactions present in the cells of

the zona glomerulosa and somewhat less in the cells of the zona fasciculata (Fig. 13). The adrenals from the rats treated with desoxycorticosterone acetate, however, showed shrinkage of the cells of the glomerulosa and a complete disappearance of lipid droplets therefrom (Fig. 14). The fasciculata appeared unaffected.



Photomicrographs of adrenal glands enucleated for 2½ months. Sudan IV and hematoxylin. $\times 200$.

FIG. 13. Gland of an untreated animal. Sudanophilic droplets present in both the glomerulosa and the outer part of the fasciculata.

FIG. 14. Gland of a rat injected for last month with 2 mg. desoxycorticosterone acetate daily. The cells in the outermost zone, the glomerulosa, are shrunken and entirely depleted of lipid droplets. Lipid remains in the cells of the fasciculata.

DISCUSSION

Regeneration of the cortex after enucleation of the adrenal

On the basis of a variety of experimental procedures, several investigators have maintained that, in the rat, new parenchymal cells of the adrenal cortex develop directly from fibroblasts in the capsule (Zwemer, Wotton and Norkus, 1938; Baker and Baillif, 1939; Salmon and Zwemer, 1941; Wotton and Zwemer, 1943; Baxter, 1946; reviewed by Jones, 1948). Gruenwald and Konikov (1944), who support this thesis with respect to many other species, have failed to detect signs of conversion of capsular cells into cortical cells in the

adrenals of rats. In the present experiments, employing a situation in which the demand for new cortical cells was very high, no signs of such conversion were seen. In the immediate post-operative period the capsule became edematous so that the fibroblasts appeared rounded. Nevertheless, the difference between fibroblasts and cortical cells remained distinct at all times. Furthermore, no mitoses were observed in the capsular cells, whereas mitoses were prevalent among the cortical cells. Since patches of cortical tissue invariably remained attached to some portions of the capsule, even after the attempt to strip it completely, proliferation of these cells seems most probably to account for all of the new parenchyma.

For the time being it seems reasonable to assume that the residual cortical cells spread out rapidly after the operation to line completely the inner border of the capsule and thus provide the "seed" for the regenerate. This conclusion agrees with the observations of Williams (1947), who obtained no indisputable evidence for the change of capsular into cortical cells in regenerating adrenal grafts. More recently, Everett (1949) has demonstrated proliferation of cortical cells when transplanted beneath the kidney capsule. Inclusion of the adrenal capsule was not essential.

During the regenerative process, cell division occurred along the entire length of the growing fascicles, rather than being limited to an outer zone. This distribution of mitotic figures suggests centripetal proliferation of the cells (Vaccarezza, 1945-6; Mitchell, 1948) rather than migration of cells from the capsular region. Nevertheless, it is an inescapable conclusion that cells which ultimately came to lie in the inner zones arose from glomerulosa cells. In an earlier paper (Deane and Greep, 1946) we stated that, on the basis of the independent reactions of the glomerulosa and fasciculata to hypophysectomy, "many facts make it seem probable that the two zones are separable and that very few cells of the glomerulosa actually migrate into the fasciculata." Apparently, under extreme circumstances at least, this conversion may occur. What proportion of the inner tissue normally derives from the glomerulosa can not be ascertained by histological techniques at present available. The use of colchicine, for instance, to determine the number of cell divisions in each zone imposes severe limitations when employed to analyze growth of cortical tissue, since this drug itself constitutes a physiological stress (Selye, 1946). Thus it undoubtedly stimulates the fasciculata at the same time that it arrests cell division, a conclusion reached by Baxter from the study of animals with and without colchicine treatment.

Restoration of secretory activity in the regenerating cortex

Two cytochemical methods were employed to indicate the secretory activity of the cortical cells. One involved the use of frozen sections prepared by a variety of methods for characterizing the lipid

droplets. Past experience has shown that the parenchymal cells in organs known to secrete steroid hormones contain droplets that are sudanophilic, Schiff-positive and autofluorescent and contain birefringent crystals (Dempsey and Wislocki, 1946; Dempsey, 1948). Whatever the actual constitution of such droplets, their presence, number and size have proved valuable in ascertaining the physiological status of the adrenal cortex (Deane, Shaw and Greep, 1948). The other method involved fixing the organ in acidified silver nitrate to reveal reducing activity presumably limited to ascorbic acid (Deane and Morse, 1948). Ascorbic acid has been shown to be especially concentrated in those organs which secrete steroid hormones, although it is not limited to them (Giroud and Leblond, 1935).

Immediately following enucleation of the adrenal, ascorbic acid disappeared entirely from the residual cortical cells. Some large lipid droplets containing coarse birefringent particles remained, however. Droplets of this character appear to signify that hormone is not being released (Deane, Shaw and Greep). Only when regeneration had proceeded for about a week did granules of ascorbic acid and small lipid droplets with fine birefringence make their reappearance. At first both were limited to the cells immediately underlying the capsule; gradually, during the succeeding 2 to 3 weeks, they reappeared in all of the new cortical tissue. The reappearance of these two types of material indicates that hormone secretion is reestablished at least within a week after enucleation, a conclusion supported in part by the observation that rats with demedullated adrenals do not require additional sodium chloride to survive, as do adrenalectomized animals (Ingle and Higgins, 1938). Indeed, Marenzi (1938) found no disturbance whatsoever in the electrolyte balance of demedullated animals.

Zonation of the regenerated gland

In view of the major part in the regeneration of the cortex played by cells originally lying in the zona glomerulosa, the possibility existed that the regenerate would function solely as glomerulosa, i.e., would secrete hormones governing the metabolism of electrolytes but not of proteins and carbohydrates. To test this hypothesis, we determined the ability of animals with newly regenerated cortices to maintain the blood sugar at normal or near normal levels during complete starvation. It is well known that in adrenalectomized rats the blood sugar level declines rapidly during fasting, leading to early death. Fasting, intact rats, on the other hand, show an initial decline in blood sugar, then a return to normal or supra-normal values (Long, Katzin and Fry, 1940). This secondary increase in the blood sugar is believed to depend on gluconeogenesis induced by 11-oxygenated corticosteroids (Ingle and Thorn, 1941; Olson, Thayer and Kopp, 1944). The response may be considered one example of the adaptation of the animal to a physiological stress (Selye).

One month after enucleation of the adrenals, rats responded to starvation in the same way as intact controls. While adrenalectomized animals died in hypoglycemic coma on the 3rd or 4th day, the normal rats and rats with regenerated adrenals survived more than 5 days and their blood sugar levels returned to 100 mg. per cent or more. This test leaves no doubt as to the ability of the regenerated cortex to influence protein and carbohydrate metabolism. Hence it is concluded that cortical cells derived from the glomerulosa can secrete 11-oxygenated hormones.

Other investigators have also found that animals with regenerated adrenals give evidence of secreting 11-oxygenated corticosteroids. Evans (1936), one of the original investigators to employ the technique of demedullation of the adrenal, reported that rats, 10 to 18 days after operation, were intermediate between intact and adrenalectomized animals in their capacity to deposit glycogen in the liver when subjected to stress. Brownell and Hartman (1948) obtained signs of unusually high secretion of cortical hormones (apparently 11-oxygenated) influencing both fat and sugar metabolism within 9 and 13 days, respectively, after adrenal enucleation. Furthermore, Ingle, Li and Evans (1946) observed glycosuria, increased blood glucose, and increased urinary non-protein nitrogen in 2 of the 3 rats given adrenocorticotropin a considerable period after demedullation of the adrenals. The remarkable thing is that we observed a normal blood-sugar response in all 10 of the rats tested, despite more severe stripping of the capsule than employed by Ingle.

Not only is the capacity of the cortical tissue to secrete both types of hormone restored upon regeneration, as indicated by the failure of rats to require extra sodium chloride and by the normal "sugar" response, but apparently the normal zonation of secretory activity is restored. In rats with regenerated adrenals, as in animals with intact glands, the administration of desoxycorticosterone acetate over a period of a month causes shrinkage of the glomerulosa cells and the disappearance of all lipid droplets. No significant alteration occurs in the inner zones. These changes in the glomerulosa have been interpreted as a "disuse atrophy" resulting from the exogenous supply of a hormone capable of substituting for the normal product of the zone (Grep and Deane, 1947). The response provides presumptive evidence that the outer rim of the cortex alone is responsible for secreting the "salt" factor in the regenerated adrenal as in the normal gland.

These observations on the restoration of functional zonation raise again the question of the basis for that zonation. Apparently cortical cells originating from the peripheral region, whether in normal organogenesis or after enucleation of the gland, have different functions at various levels from the surface. Is the formation of 11-oxygenated hormones dependent upon the presence of desoxycorticosterones in

the blood bathing the cells? Does the formation of the desoxycorticosterone-like hormones require more oxygen, more food stuffs and fewer waste products than the formation of 11-oxygenated hormones? Or, adopting either the thesis of cell migration or that of centripetal proliferation, does the age of the cell in some way influence the nature of its product? The functional zonation of the liver lobule, while less definite than that of the adrenal cortex, has compelled appreciation of the rôle of the blood supply in influencing cellular activity (Deane, 1944; Himsworth, 1948). In many ways the blood supply of the liver lobule and of the adrenal cortex is similar. More consideration of this provocative problem might prove rewarding in the understanding of the underlying biology of secretion.

SUMMARY

Following enucleation of both adrenal glands in the adult rat, proliferation of the residual cortical tissue (glomerulosa cells) restores a normal appearing cortex within a month. The regenerated cortex possesses the usual zona glomerulosa, zona fasciculata and zona reticularis.

No evidence has been found for the conversion of capsular fibroblasts to cortical cells.

Within 8 days after the operation, ascorbic acid and droplets giving the reactions characteristic of steroid-producing glands reappear in the immediate sub-capsular cells and eventually come to occupy all of the cells of the cortex.

One month after enucleation of their adrenals, rats respond in the normal fashion to a prolonged fast, i.e., the blood-sugar level declines temporarily, then returns to 100 mg. per cent within 5 days. Adrenalectomized rats succumb within 4 days in hypoglycemic coma.

The daily administration of desoxycorticosterone acetate over a period of a month causes a suppression of secretory activity in the zona glomerulosa of the regenerated gland.

The regenerated cortex apparently secretes hormones governing both salt and sugar metabolism, and the normal functional zonation is restored.

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CHANGES IN SERUM CHOLESTEROL AND CHOLESTEROL ESTERS IN ALLOXAN DIABETIC RABBITS

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THE EFFECT of diabetes mellitus on the ratio of free to esterified cholesterol of the serum has not been established (Peters and Van Slyke, 1948) although it is generally recognized that disturbances of cholesterol metabolism do occur in this condition. The usual manifestation is a hypercholesterolemia which does not seem to be related to hyperglycemia, glycosuria, or ketonuria although it is more marked in severe cases of diabetes with a tendency to ketosis and acidosis. In patients controlled either with diet or with insulin, there is usually a normal concentration of serum cholesterol; this is not invariably true as there are several reports of diabetic subjects who continued to have high postabsorptive serum cholesterol levels, even when their diabetes was well controlled (Peters and Van Slyke, 1948).

It is difficult to interpret the cause and effect relationship of cholesterol metabolism to clinical diabetes because of the complicated picture of human diabetes. Accordingly, Chaikoff and Kaplan (1933) reported a study of serum cholesterol changes in depancreatized dogs maintained on insulin wherein they showed that the cholesterol ester fraction practically disappeared while the free cholesterol fraction was only slightly lower than that of normal control animals. However removal of the pancreas imposes a multiple deficiency and it was shown by Gibbs and Chaikoff (1941) that the diet of the depancreatized dogs as well as the administration of insulin modified the level of serum cholesterol and the ester fraction.

With the discovery of Dunn, Sheehan, and McLetchie (1943) that the administration of alloxan to animals would cause the diabetic condition, a new approach for the study of the relationship of cholesterol metabolism to diabetes was opened. Moreover this procedure has the advantage of producing an essentially uncomplicated diabetic state in animals (Lukens, 1948). However, studies along this line are lacking with the exception of the report by Kendall, Meyer, Lewis, and Victor (1945) that following the injection of alloxan into rabbits

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there developed a transient period of hypercholesterolemia which accompanied the early stages of severe diabetes. This report dealt only with the changes in total cholesterol of serum, omitting any reference to the partition of free and ester cholesterol. A further study of the effect of alloxan diabetes on the changes in serum cholesterol and cholesterol esters in rabbits is reported in this paper.

EXPERIMENTAL

Six albino rabbits, each weighing about 2 kilograms, were used. The rabbits were kept on a diet of Purina Rabbit Chow and water ad lib. During a control period of two weeks, the rabbits were bled seven times; the bloods were analyzed for sugar by the micro method of Folin and Malmros (1929) and for free and total cholesterol by a modification of the method of Schoenheimer and Sperry as described by Hawk, Oser, and Summerson (1947). Samples of approximately 1.0-1.5 cc. of blood were taken both when the animals were fasted and when food was allowed in the cage overnight; inasmuch as the control values were the same under both conditions, all control blood levels of sugar and cholesterol were grouped together (Table 1).

TABLE 1. SERUM CHOLESTEROL AND BLOOD GLUCOSE LEVELS DURING CONTROL PERIOD. EACH VALUE REPRESENTS THE MEAN OF SEVEN DETERMINATIONS OVER A PERIOD OF TWO WEEKS

Rabbit	Serum cholesterol				Blood glucose mg.-%
	Total mg. %	Free mg. %	Ester mg. %	Ratio of ester to total as %	
1	56.1 ± 2.20*	23.5 ± 0.91	32.6 ± 1.89	57.9 ± 1.64	111.1 ± 2.38
2	83.4 ± 1.68	38.0 ± 1.99	45.4 ± 2.01	54.4 ± 1.55	98.1 ± 2.72
3	46.4 ± 2.36	19.9 ± 1.17	26.5 ± 1.55	57.2 ± 1.48	106.0 ± 3.67
4	64.6 ± 5.60	26.8 ± 1.92	37.8 ± 3.82	58.0 ± 1.88	101.3 ± 3.36
5	53.7 ± 1.96	23.1 ± 1.31	30.6 ± 1.57	57.1 ± 1.74	109.4 ± 2.78
6	50.2 ± 1.92	21.9 ± 0.40	28.3 ± 1.64	56.2 ± 1.26	109.5 ± 1.64
Mean	59.1 ± 2.62	25.5 ± 1.28	33.5 ± 2.08	56.8 ± 1.59	105.9 ± 2.76

* Represents the standard error of the mean calculated by the formula,

$$\sqrt{\frac{\sum d^2}{n-1}} \div \sqrt{n}$$

Without preliminary fasting, each rabbit received intravenously 200 mg. of alloxan monohydrate (Eastman Kodak Co.) per kilogram of body weight. This dose of alloxan was made up in 4 cc. of distilled water and was administered in two injections one half hour apart. Hypoglycemic convulsions developed in two animals (no. 4 and 5) in four and one half hours and were successfully controlled with intraperitoneal and subcutaneous injections of glucose solution. The four remaining animals were bled 4.5 hours after injection of alloxan and they too were given injections of glucose to prevent hypoglycemic

shock. Subcutaneous injections of glucose were continued for all animals for a period of twenty four hours. The animals were again bled twenty four hours later at which time they had access to food and water. Following this initial period of forty eight hours after the administration of alloxan, the animals were fasted overnight and samples of blood were again taken. This procedure was repeated daily for the first two weeks and thereafter at two day intervals. Thirty three days after the administration of alloxan, the rabbits were given daily morning injections of 2 units of protamine-zinc-insulin. This was increased every week by 2 units until a daily dose of 8 units was reached in five of the animals; rabbit number 2 died during the 6 unit period.

RESULTS

Within two or three days after the administration of the alloxan, all six animals exhibited hyperglycemia and in five of the six rabbits there was an increase in the total serum cholesterol. The total cholesterol continued to rise until a peak was reached between the seventh and tenth days; this peak value varied from 88.9 mg. per cent in rabbit 3 to 688 mg. per cent in rabbit 5. Thus the increases over the control values ranged from 92 per cent in rabbit 3 to 1170 per cent in rabbit 5. The serum cholesterol then began to decrease and had returned to the pre-alloxan level by the seventeenth day. The increase in total cholesterol was paralleled by an increase in the free cholesterol in all six animals; rabbit 6 in which there was no increase in total cholesterol exhibited a twofold increase in free cholesterol. These high levels varied from 40.0 mg. per cent in rabbit 6 to 525 mg. per cent in rabbit 5. The drop in free cholesterol to pre-alloxan values paralleled the decline in total cholesterol.

The changes observed in the ester cholesterol and the ester: total cholesterol ratio (expressed as per cent ester cholesterol) were in the opposite direction to the changes in the total and free cholesterol. While these latter values rose during the first week, the ester cholesterol remained the same in rabbits, 2, 3, fell sharply to 7.0 and 6.7 mg. per cent in rabbits 4 and 6, or showed a slight increase which lagged behind the increase of the free cholesterol fraction (rabbits 1, 5). Irrespective of the direction of change or lack of change in the ester fraction, there occurred a definite decrease in the ester to total ratio in all six animals. This ratio varied from a value of 3.3 per cent in rabbit 4 to 40 percent for rabbit 3 whereas the control ester: total ratios were in the vicinity of 56 to 58 per cent in all six animals (Table 1). In this first week after the injection of alloxan, five of the six animals had an ester: total ratio of less than 30 per cent while in three of the rabbits, the ratio dropped to less than 15 per cent. Following the marked drop in the ester: total cholesterol ratio, there was a return of the ratio values to control levels at about the twelfth post-alloxan day.

After the diabetes had assumed a steady state, as shown by the rather constant values for blood sugar and serum cholesterol fractions in the fasting animals from the twenty first to the thirty third days, the animals received daily injections of protamine-zinc-insulin. By the tenth day of insulin administration, there was a definite elevation of the total cholesterol levels in five of the six animals. The elevated levels varied from 63.0 mg. per cent in rabbit 3 to 240 mg. per cent in rabbit 5. Rabbit 6, which did not exhibit an increase in the total

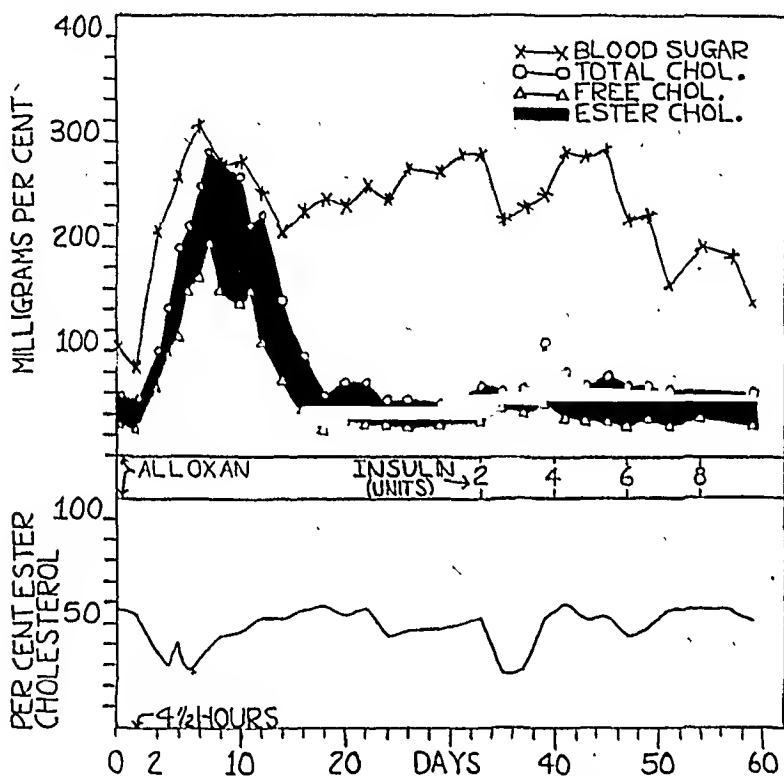


FIG. 1. Effects of alloxan and protamine-zinc-insulin on blood sugar, total serum cholesterol, free cholesterol, ester cholesterol, and ester: total cholesterol ratio. Each point represents the mean value for six rabbits.

cholesterol following alloxan administration, was the exception in this case. The increase in total cholesterol was paralleled by a comparable increase in the free cholesterol. The ester cholesterol and the ester: total cholesterol ratio dropped to extremely low levels in five of the six animals. The ester cholesterol decreased to 4.6, 5.5 and 4.6 mg. per cent respectively in rabbits 3, 4, and 6 during the first two to four days after the start of the insulin; rabbits 2 and 5 showed a moderate decrease to a level of 20 mg. per cent while in rabbit 1 there was no change in the level of the ester fraction. In all six animals there occurred a definite decrease in the ester: total ratio during the two to five day period after the institution of insulin therapy. The ester:

total cholesterol ratio varied from 5.4 per cent in rabbit 4 to 36.7 per cent in rabbit 1; in three of the six animals, the ratio was less than 20 per cent. Within six to eight days after the initiation of insulin injections, the cholesterol ester fraction and the ester: total ratio returned to the level of the control values. The results for all six animals are graphically presented in Figure 1. Rabbit 4 was deemed as being most representative of the group and the results for this animal are plotted in Figure 2.

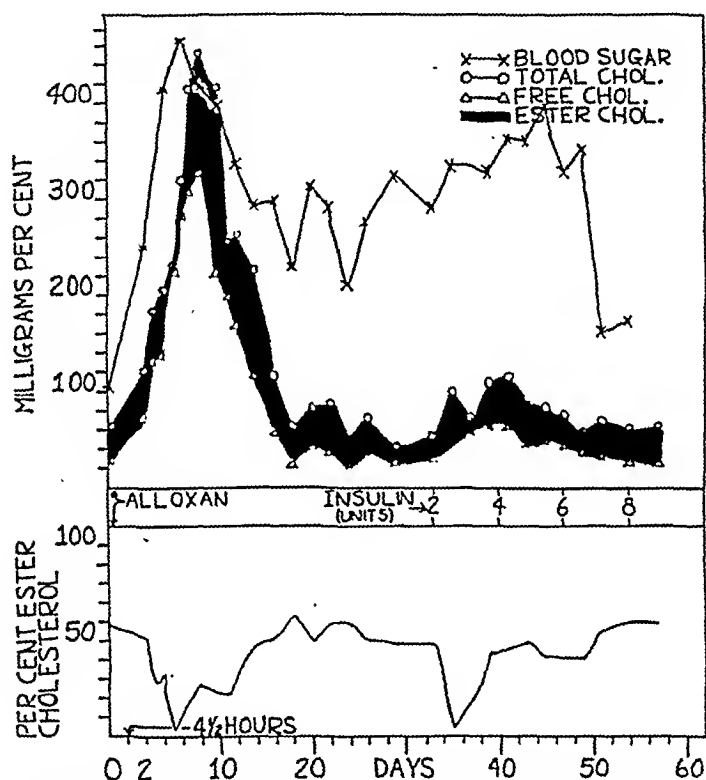


FIG. 2. Effects of alloxan and protamine-zinc-insulin on blood sugar, total serum cholesterol, free cholesterol, ester cholesterol, and ester: total cholesterol ratio in a representative rabbit (no. 4).

DISCUSSION

It is worth mentioning that with this method of administering alloxan in divided doses, a state of diabetes was produced in all six animals without a death from the usual toxic effects of alloxan (Lukens, 1948). To date we have administered alloxan according to this dosage schedule to sixteen rabbits in the course of this and other work; there have been no deaths and fifteen of the sixteen animals have been made diabetic.

The marked and sudden increase in the total cholesterol of the serum after the injection of alloxan is in agreement with the findings

of Kendall, Meyer, Lewis, and Victor (1945). Inspection of the data for the fractions of serum cholesterol shows that this hypercholesterolemia is at first due to a marked rise in the free cholesterol with the ester fraction decreasing or remaining fairly constant; this produces the marked decrease in the ester: total ratio. At six to eight days after the injection of alloxan, the increase in the total and free cholesterol has reached its peak. At this time, there is an absolute increase in the ester cholesterol and the ester: total ratio begins a return to control levels. Within twenty days after alloxan administration, all cholesterol values have returned to their pre-alloxan concentrations. There does not seem to be any relationship between the blood sugar and the serum cholesterol except for the fact that both increase within a few days after the injection of alloxan. The increase in serum cholesterol at this time, as suggested by Kendall, Meyer, Lewis, and Victor (1945), is probably due to the mobilization of lipids from the fat depots and body tissues. This is supported by the observation (Goldner and Gomori, 1943) that there is a fatty infiltration of the liver within ten to twelve days after alloxan is administered.

With the administration of insulin to the diabetic rabbits, there occurs an increase in the total and free serum cholesterol with a concomitant drop in the ester: total ratio. The changes are similar to those that occur during the first week of diabetes following the injection of alloxan. Since the increase in total serum cholesterol after the development of diabetes is thought to be due to the mobilization of cholesterol from the fat depots and other body tissues, it is suggested that subsequent to the daily injections of insulin there is a reversal of this process with the lipids decreasing in the liver and returning to the other body tissues. This is consistent with the knowledge that cholesterol in the body tissues exists as free cholesterol and not in the esterified form. Thus when the body tissues undergo a loss of cholesterol, this would be reflected in the serum as an increase in the free cholesterol. This in turn is measured as an increase in the total serum cholesterol and a decrease in the ester: total ratio. Following the increase in available free cholesterol, there is a rise in the absolute amount of serum ester cholesterol because of the presence of a cholesterol esterifying enzyme in the blood (Sperry and Stoyanoff, 1937, 1938) and in the liver (Klein, 1938; Sperry and Brand, 1941).

SUMMARY

There is an increase in the total cholesterol and free cholesterol of the serum following the injection of alloxan in rabbits. At the same time, there is a marked drop in the ratio of ester: total cholesterol of the serum, while the absolute amounts of serum ester cholesterol may decrease, or remain relatively stationary. Following the daily injection of protamine-zinc-insulin in alloxan diabetic rabbits, similar changes

in serum cholesterol and per cent of ester takes place. A possible mechanism underlying these changes is discussed.

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A SIMPLIFIED PROCEDURE FOR THYROIDECTOMY OF THE NEW-BORN RAT WITHOUT CONCOMITANT PARATHYROIDECTOMY¹

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SURGICAL thyroidectomy of the very young rat is difficult because of the size of the animal and the condition of its tissues. The operation has been performed successfully, however, by two groups of workers. Salmon (1936), who first demonstrated the feasibility of surgical excision of the thyroid gland, along with the parathyroid, in this animal as early as one or two days after its birth, employed cold anesthesia and observed survival for longer than one week in about 36 per cent of the operated rats. Scow and Simpson (1945), who employed a similar technique, with even lower temperatures for anesthetic purposes, showed survival of less than seven per cent of their totally thyroidectomized rats. In view of these difficulties, we wish to report a simplified procedure for discrete total thyroidectomy of the new-born rat without recourse to the more complicated surgical technique. This has been accomplished by the use of radioactive iodine, which, as was to be expected, can be used to destroy thyroid tissue. The purpose of the present investigation is to establish a dose that achieves complete thyroidectomy by internal radiation of the gland in the 4-12-hour-old rat and which, in addition, is of sufficiently low intensity to produce either minimal or non-deleterious effects on extrathyroidal tissues.

EXPERIMENTAL

Pregnant rats of the Long-Evans strain were fed stock diet No. 2, the composition of which has been described elsewhere (Taurog and Chaikoff, 1946). Several hours after birth, two rats of each litter were injected intraperitoneally with varying doses of carrier-free, radioactive iodine and two (one male and one female) were retained as controls. Each litter was limited to four rats. The injected rats were kept with their mothers until weaning at 28 days of age. The controls remained with their mothers for one week and were then transferred to a foster-mother. This was found necessary because the in-

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² Atomic Energy Commission Fellow.

injected rats were unable to compare with adult litter mate controls for their mother's milk. The injected rats received no special care other than that accorded to growing rats.

At various ages the rats were anesthetized by an injection of 0.5 cc. of two per cent sodium pentobarbital and eviscerated. The entire dark region (with minor variation in prearterial fascia) was excised and fixed in Davis' fluid. Representative portions of the various organs were also removed and fixed in a similar manner. The placenta was fixed in a fluid composed of the parts of a saturated solution of mercuric chloride and one part of 40 per cent formalin. All tissues were embedded in paraffin. The entire dark region was sectioned serially, and representative sections were prepared from the other organs. The placental glands were sectioned at 2-3 μ , and all other tissues at the Mallory and Coomassie¹ and each section was used for all tissues except the placenta which was stained by the method of Mallory (1935) as modified by Grisham's personal communication. Placental cell counts were made after the method of Grisham (1935); at least 200 cells per gland were counted.

RESULTS

A total of 26 days from birth to eight weeks have been injected with radioisotopes to date. Table 1 records typical observations made on the first five litters studied.

As judged by examination of serial sections of the entire dark region, no significant change was present in rats that had been injected with

TABLE 1. THE TECHNIQUE AND AVERAGE MEASUREMENTS OF RATS EXAMINED THREE MONTHS AFTER BIRTH.

Litter	Age, days	Isotope injected	Age, days	Weight, grams	Condition of thyroid	Average placental cell counts		
						Chromophobes	Luteinizing	Endothelial
1	1	SI	10	10	Completely destroyed	200 cells of total	200 cells of total	200 cells of total
	2	None	10	10	Normal	50.5	50.5	50.5
	3	None	10	10	Completely destroyed	75.4	75.4	75.4
2	1	SI	10	10	Completely destroyed	50.5	50.5	50.5
	2	None	10	10	Normal	50.5	50.5	50.5
	3	None	10	10	—	—	—	—
3	1	SI	10	10	Completely destroyed	50.5	50.5	50.5
	2	None	10	10	Normal	50.5	50.5	50.5
	3	None	10	10	—	—	—	—
4	1	SI	10	10	Some follicles with colloid absorption	50.5	50.5	50.5
	2	None	10	10	Normal	50.5	50.5	50.5
	3	None	10	10	Normal	50.5	50.5	50.5
5	1	SI	10	10	Normal and small	50.5	50.5	50.5
	2	None	10	10	Normal	50.5	50.5	50.5
	3	None	10	10	—	—	—	—

¹ See section 1.

² Data from minimum sex test.

amounts of I^{131} varying from 80 to $150\mu\text{c}$. In these rats, the region formerly occupied by the thyroid was replaced by adipose and collagenous tissue. Except for a single injected rat (Table 1, No. 3) which developed diarrhea at the age of seven weeks, all I^{131} -treated rats were in good physical condition until sacrificed. Rat No. 1 died at six days of age, probably as a result of inanition caused by inability to compete with its two normal litter mates for its mother's milk. This rat belonged to the first litter studied and its death from malnutrition led us to segregate the injected and control rats in later experiments as described above.

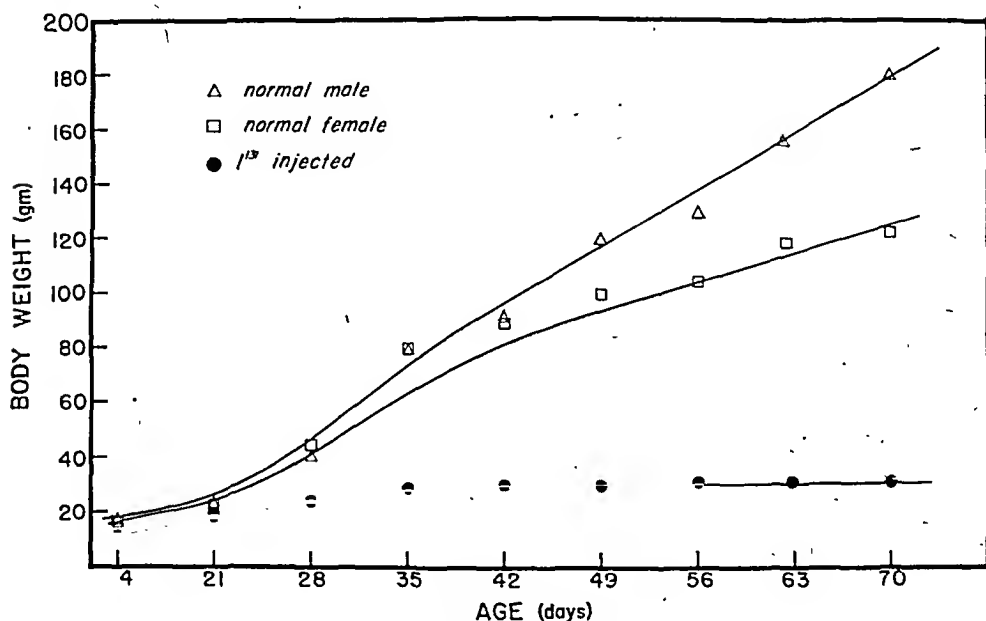


FIG. 1. Body weights of radiothyroidectomized and normal control rats.

A comparison of the growth response of rats that had been injected with $80\mu\text{c}$ of I^{131} with that of their untreated litter mate controls (male and female) is shown in figure 1. The data were obtained from litters 2 and 3 (Table 1) and each point represents the average of four measurements on as many rats. At the age of 75 days, the highest weight attained by the I^{131} -treated rats was 32 gm.; at this age, the normal male rat weighed 180 gm. (fig. 3). The oldest injected rat so far examined weighs 68 gm. at 19 weeks of age; its litter mate male control weighs 289 gm.

The roentgenogram of rat No. 3 (fig. 2), taken at the age of 75 days, clearly demonstrates the retardation of bone growth. This rat had been injected with $80\mu\text{c}$ of I^{131} . Its skeletal age, as judged roentgenographically, is that of a normal, 18 to 20-day-old rat. The criteria for estimating the bone age of this strain of rat were those employed by Scow and Simpson (1945), and Becks *et al.* (1948).



FIG. 2. Roentgenogram of a 75-day-old rat injected at birth with $80 \mu\text{c}$ of I^{131} . The skeletal age of this rat is 18-20 days.



FIG. 3. Litter mate male rats, 75 days old. The smaller was injected with $80 \mu\text{c}$ of I^{131} a few hours after birth and weighs 32 gm. Its normal litter mate weighs 180 gm.

Minute amounts of thyroid tissue were found in rats that had been injected with 60 and 40 μ c (Table 1). In those that had received 60 μ c, well defined follicles with typical epithelium were found. In one rat which had received 60 μ c of I¹³¹ (No. 5), an atypical thyroid proliferation was observed, consisting of a unilateral, partially unencapsulated mass of epithelial cells with a fetal type of architecture. The isthmus and the other lobe consisted of typical thyroid epithelium with well defined, but small, follicles which were filled with pale, eosinophilic colloid.

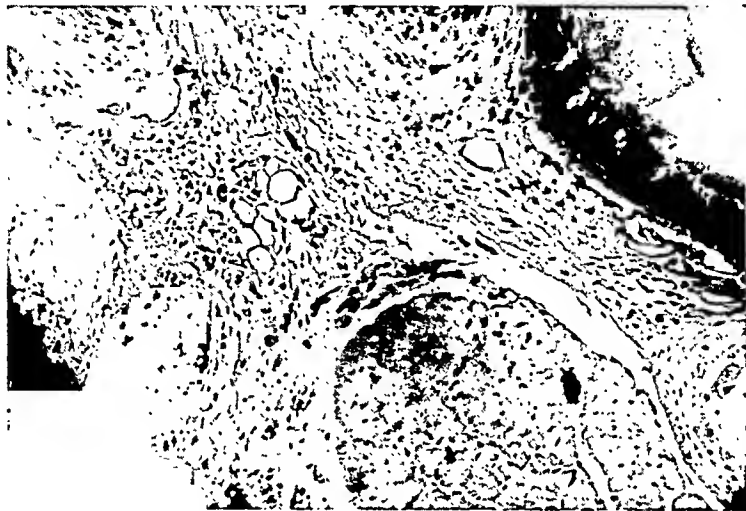


FIG. 4. Longitudinal section of the trachea of a rat that had been injected with 80 μ c of I¹³¹. Note in the lower left corner the intact parathyroid gland surrounded by adipose and collagenous tissue. Tracheal epithelium is shown in upper right hand corner. (Haematoxylin and eosin; 10 μ ; $\times 125$.)

The pituitary changes found in the I¹³¹-treated rats are recorded in Table 1. The acidophils were almost completely degranulated in rats in which no thyroid tissue was detected by histological examination of serial sections of the neck region (rats Nos. 2, 3, and 4). Interestingly enough, in rats in which as few as 14–16 surviving follicles remained, degranulation of the acidophils was only slight. The presence of typical thyroidectomy cells (large, vacuolated basophils) were observed in all I¹³¹-treated rats; this finding indicates that even the lowest dose of I¹³¹ injected had resulted in sufficient thyroid damage to induce a thyroxine deficiency.

No pathological changes were observed in liver, kidney, gastrointestinal tract, and heart. Evidence of damage (loss of ciliation and nuclear pyknosis) were found in the tracheal epithelium in the region adjacent to the destroyed thyroid gland. A few pyknotic and some atypical enlarged hyperchrorratic chondrocytes were also found in the adjacent cartilage.

Intact parathyroids were found in all rats in which complete de-

struction of the thyroid gland had occurred (figs. 4 and 5). Aside from a few pyknotic cells in the peripheral portions, and some increase in interstitial connective tissue and a thickened capsule, the parathyroids appeared essentially normal histologically. No tetany was observed in any rats.

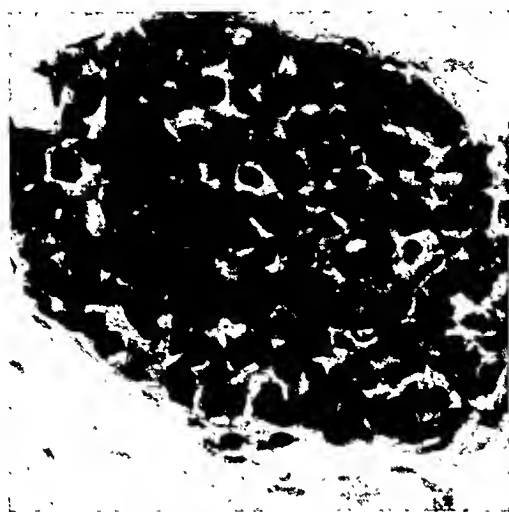


FIG. 5. Parathyroid gland shown in fig. 4, $\times 500$.

COMMENT

No single criterion can be used to establish completeness of thyroidectomy. For this reason, evidence as to whether the thyroid gland had been completely destroyed in new-born rats by the injections of I^{131} was sought by a study of growth response, bone age, and anterior pituitary cytology in addition to histological examination of serial sections of the entire neck region. As judged by these criteria, it would appear that total thyroid destruction can be achieved in the new-born rat by the injection of 80 or more μc of I^{131} .

Griesbach (1943, 1945, and 1946) has called attention to the value of quantitative cytological study of the adenohypophysis for the estimation of thyroid function. Previous work by Hohlweg and Junkmann (1933) and Lebedewa (1936) pointed to the fact that complete absence of thyroid tissue leads to complete degranulation of the acidophils of the rat anterior pituitary. In a series of studies, Griesbach (1943, 1945, 1946) correlated the percentage of acidophils with the level of circulating thyroid hormone and demonstrated that only in the case of total thyroidectomy was degranulation of the acidophils complete. This work has been confirmed in this laboratory (unpublished observations) and has now been observed in those rats which were *radiothyroidectomized*. In those rats which were found to have no thyroid

tissue upon complete serial section of the neck region and which had, in addition, the characteristics of new-born thyroidectomies as described by Salmon (1936) and Scow and Simpson (1945), among others, the acidophil count in the pituitary was found to be in the region of one per cent or less. In those rats which were found to contain only a few surviving follicles, however, the acidophils were present in from 15-40 per cent of the total cell count. Although the presence of the acidophils in the degranulated condition can be demonstrated by special techniques (Severinghaus, 1932), or by regranulation experiments with thyroxine (Griesbach, 1946), the usual practice of including degranulated acidophils in the total chromophobe count has been followed in the present study.

Aside from its simplicity, radiothyroidectomy has certain advantages over the surgical procedure for excision of the gland. These are: low mortality (one of the 28 I^{131} -treated rats died); no adverse bone effects due to refrigeration anesthesia (Scow and Simpson, 1945; Scow, 1949); and the relative ease of maintenance of the treated animals. An interesting feature of this procedure is the survival of the parathyroids even when the dose of I^{131} injected is large enough to wipe out all thyroid tissue completely.

ACKNOWLEDGMENT

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SPECIFICITY OF THE INTRAUTERINE TEST FOR PROGESTERONE¹

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IN AN EARLIER paper (Hooker and Forbes, 1947) a bioassay that detects 0.0002 μ g of progesterone was described. The sensitivity of the test readily permits the measurement of levels of this hormone in blood (Hooker and Forbes, 1947, 1949) and other body fluids, and offers promise of being useful in investigating several aspects of the metabolism of progesterone. To be of more than seriously limited value, however, an assay must be more or less specific, irrespective of its sensitivity. It has already been reported (Hooker and Forbes, 1947) that estrone, α -estradiol, desoxycorticosterone acetate, and testosterone failed to elicit the reaction used as a test for progesterone. The specificity of the test has been examined further by administering several additional substances.

All of the substances tested are listed in Table 1. Included are (1) compounds reported to duplicate the action of progesterone in other tests, (2) compounds considered to be metabolites or possible precursors of progesterone, (3) compounds closely related to progesterone chemically, and (4) compounds that might be expected to accompany progesterone in biological material.

The assay involves injection of a solution of the material to be tested directly into a segment of the uterus of an ovariectomized mouse. The response to progesterone consists of transformation of some of the endometrial stromal nuclei, which in the castrate are

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Progesterone, desoxycorticosterone acetate, dehydroisoandrosterone, anhydrohydroxyprogesterone, Δ^4 -pregnenolone, pregnanedione, allopregnanedione, allopregnanolone, estradiol dipropionate, and estradiol benzoate were furnished by Dr. Erwin Schwenk of the Schering Corporation; desoxycorticosterone, by Dr. Albert Segaloff; α -estradiol, androsterone, testosterone, and testosterone propionate, by the late Mr. Robert A. Mautner, and methyl testosterone, cis-testosterone, testosterone dipropionate, by Dr. Ernst Oppenheimer, of Ciba Pharmaceutical Products, Inc.; pregnanediol, by Dr. A. Stanley Cook of Ayerst, McKenna and Harrison, Ltd.

The adrenal extracts were obtained by Dr. Abraham White from The Wilson Laboratories. The oily extract was listed as containing 40 rat units (Survival-Growth) in each ml., and 1 ml. of the undiluted aqueous extract was listed as having been derived from 75 gms. of suprarenal tissue.

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shrunken, dense, and fusiform, into plump, slightly elongated oval nuclei with conspicuous nucleoli and fine, evenly dispersed chromatin particles. The details of the method have been described before (Hooker and Forbes, 1947), and need not be repeated here. With three exceptions all of the substances tested were dissolved in sesame oil. Water was the solvent for the ascorbic acid and the aqueous adrenal extract, and an unidentified oil was the solvent for the oily

TABLE 1. SUCCESS (+) OR FAILURE (—) OF COMPOUNDS IN EVOKING A NUCLEAR RESPONSE IDENTICAL WITH THAT PRODUCED BY PROGESTERONE

	mg./I.U. (Selye & Masson)	Intrauterine injection, micrograms		Subcutaneous administration, milligrams	
		—	+	—	+
Progesterone	1	.0001	.0002	0.375, 0.875, 0.875	1.5, 0.75 0.375, 0.375
Anhydrohydroxyprogest. (Ethinyl testosterone)	10	Less than .006		Small amt. from pellets (2 mice)	
Desoxycorticosterone acetate	10	6, 3, 0.6		5, 2.5, 1.25	5, 2.5
Desoxycorticosterone		6, 3, 1.5			
Methyl testosterone	30	15, 3, 0.6		25, 12.5, 6.25, 1, 9.5, 3, 125, 1.563, 1, 0.75,	25, 12.5, 6.25, 6.25
Testosterone prop.	50	15, 15, 3, 3, 0.6, 0.6			
Testosterone diprop.		3, 0.6, 0.12			
Δ^4 -pregnenolone	40-100	5.7, 2.9, 1.4		5, 5, 2.5	
Testosterone	140-200	0.48, 0.24, 0.16, 0.12		2.5, 2.5, 2.5	
Cis-testosterone		Approx. 8, 8, 4, 4			
Pregnanediol	0	Unknown concn.			
Pregnanedione	0	3, 1, .005	127, 67		
Allopregnanedione		6, 3, 1, .005	127		
Allopregnanolone		12, 6, 3, 1, 0.005			
Androsterone	0	6, 6, 3, 1.5			
Dehydroisoandrosterone	0	6.3			
Cholesterol	0	5.2, 2.6, 1:3			
Oily adrenal cortical ext.		Undild., $\frac{1}{2}$ dild.			
Aqueous adrenal cortical ext.		$\frac{1}{2}$ dild.	Undild.?		
Estrone		0.6, 0.3, 0.15			
Estradiol	0	.000000023- .00075 (36 mice)			
Estradiol benzoate				.0488, .0488, .0488	
Estradiol dipropionate				.0001 (6 mice)	
Ascorbic acid in water		171, 85.5			

adrenal extract. In each instance the volume of vehicle injected was 0.0006 ml. The quantities in micrograms of each substance given are shown in the table.

As indicated in the table, progesterone was the only one of the 24 substances that provoked changes in the endometrial stromal nuclei that clearly satisfied all of the criteria of the assay. Many of the other substances provoked changes of various kinds in the stromal nuclei, most of which were quite distinct from the changes elicited by progesterone. Pregnanedione in amounts of 12 and 6 μ g, allopregnanedione in a dose of 12 but not 6 μ g, and the undiluted aqueous adrenal extract evoked changes that were sufficiently similar to those of progesterone that the reactions had to be recorded as questionable positives; these responses, however, could not be read as clear-cut positive reactions.

Inasmuch as quantities of the order of 30,000 to 60,000 times the

effective dose of progesterone gave but a questionably positive reaction, there can be some doubt that pregnanediol and allopregnanediol duplicated the action of progesterone in the test. The questionably positive effect of the undiluted aqueous adrenal extract could possibly have been due to a small quantity of progesterone in the extract. Beall (1938) and Von Euw and Reichstein (1941) have isolated progesterone in adrenal extracts, and a computation suggests that the assay here would be positive if the extract tested contained but 1 mgm. of progesterone for each 225 kg. of adrenal tissue processed. It is possible, however, that one of the "true" cortical steroids almost duplicated the effect of progesterone in the test.

Whether the test is completely specific for progesterone can probably never be ascertained rigorously; an almost endless series of compounds in a variety of doses would have to be examined. The group of substances tested, however, include most of the substances that offer a possibility of being a source of error, and only progesterone gave an unequivocally positive reaction.

In addition to intrauterine injection, certain of the substances were also given systemically, as shown in the table. The anhydrohydroxyprogesterone was given in the form of a subcutaneous pellet (one mouse) and an intrasplenic pellet (one mouse), and the animals were killed for study after 14 days. The other compounds given systemically were dissolved in sesame oil and injected subcutaneously in five or six daily doses. The total quantity administered is listed in the table.

Testosterone propionate in amounts of 6.25 mgm. or higher regularly and desoxycorticosterone acetate in amounts of 2.5 mgm. and higher sometimes duplicated the action of progesterone on the stromal nuclei (Hooker, 1945). The other compounds in the quantities administered failed to reproduce the action of progesterone. Neither desoxycorticosterone acetate nor testosterone propionate gave a positive test when administered topically, and neither was effective when given systemically until 16.6 and 6.6 times the minimal effective amount of progesterone was administered.

These observations seem to be more consistent with the possibility of conversion of a fraction of the subcutaneously injected compound into progesterone than with progestational activity being an inherent property of these two compounds as such. It would be interesting to know whether the several compounds found by Selye and Masson (1943) to exhibit luteoid activity when given systemically to rabbits are also effective when applied directly to the endometrium of the rabbit. The multiple and overlapping actions of many steroids as studied by many investigators could conceivably be the result of metabolic interconversion (large quantities are almost always necessary) rather than inherent pharmacologic properties of the various compounds.

Questions of this sort are important in determining what chemical configuration is basic to any particular action of a steroid hormone. The limited data presented here perhaps suggest that the molecular morphology of progesterone is necessary in this test, and that modifications, however slight, abolish the action studied.

SUMMARY

The specificity of a sensitive bioassay for progesterone that depends upon intrauterine injection in mice has been examined by administering 24 substances. Included were compounds that are progestational by other tests, compounds that may be metabolites or precursors of progesterone, and compounds that might be expected to accompany progesterone in biological materials. Only progesterone gave a clearly positive reaction. Pregnanedione and allopregnanedione in large doses and an undiluted aqueous adrenal extract provoked questionable positive responses.

Certain of the compounds were also given systemically. Desoxycorticosterone acetate and testosterone propionate in large doses duplicated the action of progesterone upon the endometrium.

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THE EFFECT OF VARYING LEVELS OF THYROIDAL STIMULATION ON THE ASCORBIC ACID CONTENT OF THE ADRENAL CORTEX

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THERE IS considerable evidence suggesting an interrelationship between the thyroid gland and the adrenal cortex. Hoskins (1910), Herring (1917), Cohen (1935), Schmidt and Schmidt (1938), Deane and Greep (1947), and others have reported that following the administration of thyroidal substances to animals, the adrenals enlarge, the hypertrophy being restricted to the cortex.

Leblond and Hoff (1944) noted a decrease in the size of the adrenals in rats receiving goitrogenic sulfa drugs or thiouracil. This was confirmed by Baumann and Marine (1945) when they noted involution of the adrenals to half their former size in rats fed thiouracil for four months.

From these reports it seems apparent that the functional activity of the adrenal cortex is interrelated in some manner with the thyroid secretion level. Very little information has been reported however, that specifically relates the adrenal cortical secretion to the thyroid status.

It has been demonstrated recently by Long (1947) and Sayers and Sayers (1948) that the adrenal cholesterol and ascorbic acid concentrations provide a reliable index of the functional activity of the adrenal cortex. In the work to be reported, the ascorbic acid content of the adrenals was used to determine the influence of varying degrees of thyroidal stimulation on adrenocortical function in the male white rat.

EXPERIMENTAL

Except as otherwise stated, male rats of the Michigan State College strain were used. After a given experimental treatment, the animals were killed by decapitation, and the adrenals were dissected out, trimmed cleanly, and weighed to the nearest tenth of a milligram. Following this, they were placed in 50 cc. centrifuge tubes containing about 3.5 gm. of coarse sand saturated with 1 cc. of 3% metaphosphoric acid that was prepared by the method of Briggs (1938). The

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glands were macerated by gentle grinding with a glass rod. The resulting mass was washed with two 10 cc., and one 5 cc. portion of 3% metaphosphoric acid, and centrifuged after each addition of acid. The resulting supernatant liquid was freed of turbidity by mixing with celite and subsequent centrifugation.

A twenty cc. aliquot of the clear extract was then adjusted to a pH of 3.0–3.5 with 10 cc. of a sodium citrate buffer. The ascorbic acid content of the buffered extract was determined by the method of Mindlin and Butler (1937) as modified for plant and animal tissues by Bessey (1938).

The Response of the Adrenals to a Constant Level of Thyroidal Stimulation as a Function of Time

It has been demonstrated by Long (1947) and Sayers and Sayers (1948) that time relationships are very important in the response of

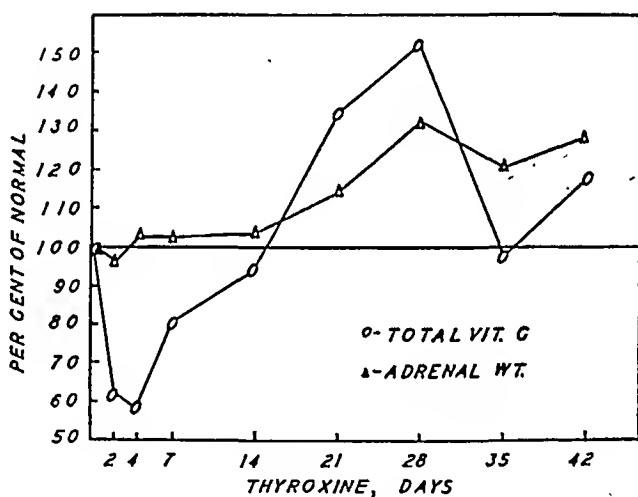


FIG. 1. Changes in the weight and ascorbic acid content of the adrenals brought about by a daily dosage of 20 μ g. of thyroxine per 100 gm. body weight during a six-week period.

the adrenals to acute stress. This experiment was designed to investigate the response of the adrenals to the more prolonged stimulation of thyroxine injections.

Eight groups of at least 5 rats each were injected daily with a solution of d, l-thyroxine at a dosage of 20 μ g. per 100 gm. body weight, this dosage being approximately 5 times the estimated thyroid secretion rate of this strain of rats (Reineke, unpublished data).

Four groups of controls were used. Group 1 was killed at the start of the experiment, and the second, third, and fourth groups, were killed after 3, 4, and 6 weeks respectively. The last three groups received daily injections of 0.1 cc. of physiological saline solution per 100 gm. body weight.

Although there was some variation between the different control groups, no consistent trends in either adrenal weight or ascorbic acid were noted. Consequently the values for adrenal weight and ascorbic acid, respectively, were pooled and are shown graphically in figure 1 as the base line or 100%. The mean control adrenal weight with standard deviation was 42.0 ± 5.98 mg. The value for ascorbic acid was 194.8 ± 52.7 ug. All the values in the experimental groups were calculated as percentages of this base.

Values obtained 2 and 4 days after thyroxine injections were begun showed a pronounced decline in the ascorbic acid content of the adrenals (Fig. 1). This was followed by a return to normal levels and finally by considerable overcompensation, as shown by the pronounced rise above normal after 28 days of stimulation. Coincident with the leveling off of the adrenal weight at a point considerably above normal, the ascorbic acid content showed a trend toward normal values.

Effect of Varying Levels of Thyroidal Stimulation on Adrenal Weight and Ascorbic Acid Content

This experiment was designed to determine the effects of varying levels of thyroidal stimulation on the response of the adrenals at 4 weeks.

Koger and Turner (1943) showed that dosages of thyroprotein in the feed, ranging from 0.01% to 0.16%, produced noticeable and largely adverse effects in male rats with regard to growth, the higher levels showing a definite toxic effect. The effects on the adrenals varied with two strains of rats employed. One strain showed hypertrophy of the adrenals which "paralleled roughly the level of thyroprotein fed." Females of the other strain showed this hypertrophy but, surprisingly, the males did not show an increase in adrenal weight, and with some of the dosages used, the adrenals showed a statistically significant decrease in size.

In this experiment a thyroactive iodinated protein "Protamone"² was fed in dosages corresponding to the first five levels used by Koger and Turner (1943) which ranged from 0.01% to 0.16% mixed in the feed and given *ad libitum*. After 4 weeks of treatment (Table 1, Part 1) there was no significant trend in either the adrenal weight or ascorbic acid content of any of the experimental groups when compared with the controls. This would indicate that the dosage range was too low to evoke a response from the adrenals in this strain of rats.

In order to determine the effect of higher levels of thyroidal stimulation, 5 groups of rats were placed on daily injections of thyroxine ranging from 5 ug. to 80 ug. per 100 gm. body weight for a period of 28 days. Five ug. of thyroxine per 100 gm. body weight had no significant effect on either adrenal weight or vitamin C content after

² Kindly supplied by the Cerophyl Laboratories, Kansas City, Missouri.

four weeks of treatment. At dosages of 10 and 20 ug. per 100 gm. body weight, there was a progressive increase in the size and ascorbic content of the adrenals. Forty ug. of thyroxine per 100 gm. body weight caused no further increase in either adrenal weight or ascorbic acid content, when compared with the group receiving 20 ug. daily. When the dosage level of thyroxine was increased to 80 ug. per 100 gm. body weight, however, there was an increase of 52% in weight, and 97% in ascorbic acid content of the adrenals of the hyperthyroid rats when compared with the controls.

It has been reported by numerous authors that the adrenals of male animals are smaller than those of the female. To explore the possibility of sex differences in vitamin C in this experiment, a group of 8 females of approximately the same age, were killed concurrently with the normal controls (Table 1, part 2). It was found that the female adrenals were significantly heavier than the males. The ascorbic acid content was also slightly higher than that of the males, but this difference was not statistically significant.

Determination of the Increase in the Secretion Rate of the Adrenal Cortex Due to Hyperthyroidism

There is extensive literature reporting that the administration of adrenocortical extracts, and hormones structurally related to those secreted by the adrenal cortex, will produce adrenocortical atrophy. (Selye, 1940; Carnes, *et al.* 1941; Ingle, 1939). Ingle *et al.* (1937) postulated that when there is an amount of cortin present in the body above physiological requirements, the output of adrenocorticotrophic hormone is suppressed. Ingle (1938) further observed that when the hypophysis is absent, and the size of the adrenals is maintained with adrenocorticotrophic hormone, cortin has no effect on the adrenals.

From these facts a method for estimating the increase in the adrenocortical secretion rate in hyperthyroid rats may be suggested. As has been shown in the preceding experiment, the maximum response of the adrenals to a dose of 20 ug. of thyroxine per 100 gm. body weight per day, is seen at the fourth week. It was thought that if this response could be prevented, wholly or in part, by the simultaneous administration of adrenocortical hormone, the dose of hormone necessary to accomplish this would be an indication of the increase in the secretion rate of the adrenals caused by this degree of thyroidal stimulation. In addition this would be *prima-facie* evidence that the adrenals are in a state of hypersecretion in a hyperthyroid animal. The hypersecretion is regarded as evidence for an increased requirement for the adrenocortical hormone in these animals. Evidently more adrenocorticotrophin is secreted when thyroxine is administered, since the adrenals are enlarged.

TABLE 1. EFFECT OF VARYING LEVELS OF THYROIDAL STIMULATION ON ADRENAL SIZE AND ASCORBIC ACID CONTENT DURING A 28 DAY PERIOD

Part 1. Effect of Feeding Protamone at Physiological Levels of Stimulation					
Group	No. of animals	Body wt. gm.	Adrenal wt. mg.	Total vit. C. ug.	% Protamone in feed
1	9	238±24.0	38.5± 5.3	180.4±22.6	0.01%
2	8	231±22.0	35.0±11.8	142.3±27.8	0.02%
3	9	231±36.0	41.3± 4.4	186.7±26.4	0.04%
4	10	237± 7.0	43.7± 2.2	208.9±40.7	0.08%
5	10	219±23.0	40.3± 7.5	185.9±46.2	0.16%
Controls	9	243±26.0	39.9± 5.2	170.5±27.8	No treatment
Part 2. Effect of Injections of Thyroxine at High Dosage Levels (ug. Thyroxine/100 gm. Body Wt.)					
1	4	257±12.0	42.3± 3.3	204.6±26.4	5
2	4	251±26.0	49.8± 3.3	246.5±16.0	10
3	5	271±19.0	55.5± 6.3	296.0±15.4	20
4	5	236± 9.0	55.3± 5.5	282.4±38.5	40
5	5	223±19.0	63.8± 5.9	401.7±31.3	80
Pooled Controls	22	260±20.0	42.0± 5.9	194.8±52.7	0.1 cc. Physiological Saline/100 gm. Rat
Normal Females	8	220±19.0	52.4± 6.8	229.9±22.4	No treatment

TABLE 2. THE INHIBITORY EFFECT OF ADRENOCORTICAL HORMONE ON THE ADRENOCORTICAL HYPERTROPHY ELICITED BY THYROXINE STIMULATION

Group*	Body wt. gm.	Adrenal wt. mg.	Total vit. content ug.	Treatment**—units of Eschatin/day
1	276±12.7	52.8±5.9	289.6±30.1	10
2	262±11.7	46.9±4.5	249.2±11.7	20
3	261± 9.6	46.8±5.7	261.2± 9.6	40
4	271±19.0	55.5±6.3	296.0±15.4	No Eschatin.

* Five rats were included in each group.

** Twenty ug. thyroxine was given daily per 100 gm. body weight for 28 days. During the last 8 days of this period Eschatin was given concurrently with the thyroxine in the amounts specified.

Accordingly, the adrenocortical extract "Eschatin"³ was injected into three groups of five rats each, which had received injections of 20 ug. of thyroxine per 100 gm. body weight daily for 20 days. Group 1 received 10 units of Eschatin daily. Groups 2 and 3 received 20 and 40 units per day, respectively. The animals were kept on simultaneous injections of thyroxine and cortical extracts for 8 days, after which they were sacrificed, and the adrenals were removed and analyzed for ascorbic acid.

Group 1 which received 10 units of cortical extract per day showed

³ We are indebted to Dr. H. O. Von Rosenberg, Parke, Davis and Company, Detroit, Michigan, for supplying the cortical extract, "Eschatin," used in these experiments.

no significant decrease in either adrenal weight or ascorbic acid content, when compared with the group receiving the same amount of thyroxine alone for 28 days.

The two groups receiving 20 and 40 units of cortical extract per day, respectively, in addition to thyroxine injections, showed no significant differences when compared with each other in either adrenal weight or ascorbic acid. When these two groups were compared with the group receiving thyroxine alone for 28 days, a highly significant decrease both in adrenal size and ascorbic acid was observed.

It seems evident from these data, that the increase in the secretion rate of adrenocortical hormones in rats receiving 20 ug. of thyroxine per 100 gm. body weight per day, is between 10 and 20 dog units per day. Above the dosage of 20 units per day, additional adrenocortical hormones did not show a perceptible effect under our experimental conditions.

DISCUSSION

It has been demonstrated in the experimental work of this report that time relationships are very important in the response of the adrenals to prolonged stimulation with thyroxine. Assuming that the vitamin C content of the adrenals is an index of secretion, it would appear that the adrenals are discharging increased amounts of cortical hormones into the blood stream in the earlier stages of induced hyperthyroidism. At this stage the adrenal cortex has only a normal amount of secretory tissue present. Consequently, the discharge of hormone exceeds its rate of synthesis in the gland as evidenced by the pronounced decline in ascorbic acid content after 2 and 4 days of stimulation. Concurrently with the development of more secretory units in the adrenal at the third week, as shown by increased weight, there is an increase in the vitamin C content above normal values. This means that not only is there an increased rate of secretion, but also an increased rate of hormone synthesis.

At four weeks there is an oversupply of adrenocortical hormones, and when these are discharged, they inhibit further secretion of adrenocorticotrophic hormone by the anterior pituitary. This is followed by a decline in adrenal vitamin C and a noticeable decrease in adrenal weight. At the sixth week, a balance may be presumed to be established, in which the size of the adrenals and the rate of hormone synthesis and secretion is adequate for the degree of thyroidal stimulation to which the animal is being subjected. However, the mechanism whereby this balance is brought about remains to be elucidated.

Evidence is advanced to show that the level of thyroidal stimulation determines to some extent, the size and secretory activity of the adrenal cortex. There is a rather narrow dosage range in which thyroidal stimulation has no demonstrable effect on the adrenals. This range may be considered to be the "Physiological Range," or the dosage approximating the animal's own thyroid secretion rate.

When measuring the hormonal activity of the adrenal by ascorbic

acid assay, it might be questioned whether an increased amount of vitamin C in the adrenal means increased synthesis and subsequent liberation of adrenocortical hormones, or whether the increase in ascorbic acid content is merely indicative of increased storage. In favor of the first interpretation is the fact that the initial response of the adrenal to adequate thyroidal stimulation is a decrease in ascorbic acid. Only when enough secretory units are laid down so that the adrenals can balance the demand for cortical hormones does the ascorbic acid content show an increase.

Strong evidence that the adrenals of a hyperthyroid rat are in a state of hypersecretion is provided by the fact that 10-20 dog units of cortical hormone are required daily to bring the pituitary-adrenal mechanism back into normal balance.

SUMMARY

The effect of thyroidal stimulation on the adrenal ascorbic acid content and adrenal weight of the male white rat has been studied. The ascorbic acid content of the adrenal decreases to minimal values after 4 days of thyroxine administration. This is followed by progressive increases, both in adrenal weight, and ascorbic acid content which reach a maximum at 4 weeks. Following this, there is a general leveling off at the fifth and sixth weeks. The response of the adrenals to different adequate dosage levels of thyroxine is one of a progressive increase in size and ascorbic acid content at 4 weeks. The increased secretion rate of the adrenals in a hyperthyroid rat has been estimated to be equivalent to the amount falling between 10 and 20 dog units of adrenocortical hormones.

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COMPARATIVE ORAL THYROXINE-LIKE ACTIVITY OF NATURAL AND SYNTHETIC THYROPROTEINS AND OF DL-THYROXINE STUDIED WITH THE GOITER PREVENTION METHOD¹

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LOS ANGELES

INTRODUCTION

IN A PREVIOUS report (Frieden and Winzler, 1948) it was concluded that parenterally administered natural thyroid proteins exert greater thyroxine-like activity than could be attributed to their L-thyroxine content. Synthetic thyroproteins, on the other hand, showed only one-half the parenteral activity expected from their L-thyroxine analysis. The convenience, precision (see Hutchison, 1948) and sensitivity of the goiter prevention method in the physiological range of thyroxine dosage suggested its application to the determination of the relative oral thyroxine-like activity of natural and synthetic thyroproteins and of DL-thyroxine. This is a factor of some importance in research and in thyroid therapy.

METHODS AND MATERIALS

The procedure for the biological assay for thyroxine-like activity was essentially the same as that described earlier. The thyroid glands were weighed on removal from the test animal after 15 days on a stock diet containing 0.3–0.5% thiouracil incorporated into a stock diet with a daily oral administration of the test substance. The biological activity of the preparation was estimated to the nearest 0.05 γ from a plot of the thyroid weight against parenteral DL-thyroxine controls for each individual experiment. In these experiments male rats weighing 100–200 gm. were used, and all test materials were orally administered except for certain intraperitoneally injected DL-thyroxine controls.

The thyroactive substances used in this study were the various natural thyroid preparations and the synthetic thyroproteins described previously by Frieden and Winzler (1948). The test materials were dissolved or suspended

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in an aqueous solution containing 10% glucose, 0.50% chlorobutanol and 0.10% NaHCO_3 . DL-thyroxine was dissolved in a minimum amount of 0.1 N NaOH, the pH adjusted to 8.0 ± 0.5 and the solution made up to volume with the above glucose solution in the case of the solutions used for oral administration or with distilled water for those solutions used for intraperitoneal injection. Volumes of approximately 0.10 ml. per 100 gm. body weight of these mixtures could be satisfactorily administered once daily by mouth through a 0.25 ml. syringe fitted with a polished blunt 20 gauge needle.

RESULTS AND DISCUSSION

The results obtained in this survey of the oral activity of various thyroactive materials are summarized in Table 1. Sample data from one of six independent experiments is shown in Figure 1. These data show that under these conditions, orally administered thyroxine is about one-half as active as when given parenterally. It is emphasized that the doses of thyroxine employed in these experiments were in

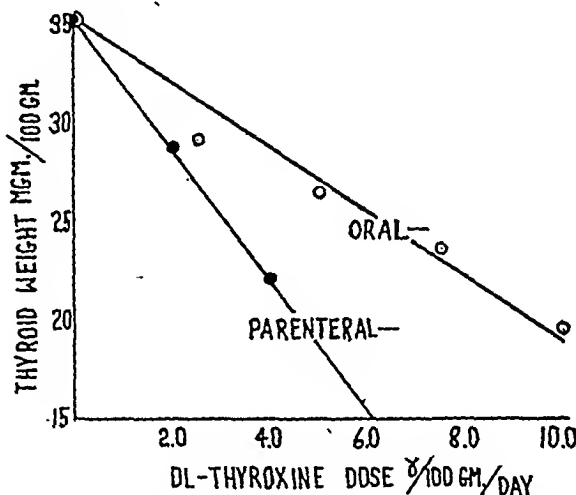


Fig. 1. Relative activity of orally and parenterally administered DL-thyroxine as indicated by a plot of DL-thyroxine dose against thyroid weight. Groups of six 100–200 gm. male rats were maintained on a 0.3–0.5% thiouracil diet for 15 days during which time they were given daily doses of DL-thyroxine by the indicated route.

the physiological range and are not directly comparable to most previous work² in which much greater doses were administered to produce significant effects on oxygen consumption and other less sensitive methods for measuring thyroxine-like activity. One factor contributing to the high oral activity of DL-thyroxine in these experiments is the fact that the substance was in solution when given. While the thyroxine was probably precipitated at the pH of the stomach,

² Previous work regarding the relative oral activity of thyroactive materials is summarized in a number of texts including those of Kendall (1929), Harington (1933), Means (1937), Elmer (1938), and Salter (1940).

TABLE 1. SUMMARY OF EXPERIMENTS ON THE THYROXINE-LIKE ACTIVITY OF NATURAL AND SYNTHETIC THYROPROTEINS WHEN ADMINISTERED ORALLY

0.3%-0.5% thiouracil diet plus	Per cent total iodine	Per cent thyroxine	No. of groups tested	Total no. of animals tested	Range of dosage	Biological Response ¹	Per cent of parenteral activity ²
					γ -DL- thyroxine or equivalent per 100 gm. wt.	γ -thyroxine in dose	
DL-thyroxine	65.4	100.0	9	54	2.5-10.0	0.50 \pm 0.029 ²	49
Desiccated thyroid—1	0.52	0.26	6	35	0.75- 2.0	2.85 \pm 0.160	71
Desiccated thyroid—2	0.64	0.31	4	25	0.75- 1.0	2.65 \pm 0.24	72
Product A	0.99	0.46	3	15	1.00- 2.0	3.00 \pm 0.105	98
Product B	0.27	0.13	3	19	1.00- 2.0	3.10 \pm 0.110	71
Synthetic thyroprotein							
—1	10.7	2.6	4	22	12.0-30.0	0.143 \pm 0.0012	13.6
—2	8.6	2.9	1	6	20.0	0.120 \pm 0.018	14.1

¹ Estimated to the nearest 0.05 γ from parenteral DL-thyroxine dose-response curve for particular experiments.

² Standard error of the mean.

³ Calculated from the expression: $\frac{\text{oral biological response}}{\gamma\text{-thyroxine in dose}} \times 100 / \frac{\text{parenteral biological response}}{\gamma\text{-thyroxine in dose}}$
the latter ratio from a previous publication (Frieden and Winzler, 1948).

the flocculent nature of such a precipitate would have facilitated resolution in the duodenum.

Table 1, column 6, lists the ratio of the biological activity of the indicated thyroprotein to the chemical thyroxine content of the dose. If it be assumed that L-thyroxine possesses twice the biological activity of DL-thyroxine, this ratio should have a maximum theoretical value of 2.0. The results obtained with natural thyroid proteins indicate that even when given orally, these materials exhibit ratios significantly greater than 2.0, averaging about 2.9.³ Thus desiccated thyroids 1 and 2 and commercial product B gave ratios of 2.85, 2.65 and 3.10 respectively which represented 71%, 72%, and 71% of their parenteral ratios as indicated in column 7. Commercial product A, with the highest thyroxine content and greatest solubility of any of the natural thyroid proteins, retained 98% of its parenteral activity. Even larger ratios of 5.8 result from calculating the ratio of the biological activity in terms of *oral* DL-thyroxine activity to the chemical thyroxine content. Since it is reasonable to assume that thyroglobulin cannot be absorbed intact, the above data suggests a high order of digestibility of natural thyroid proteins.

Synthetic thyroproteins, in contrast, were considerably less potent orally than parenterally. Two preparations showed only about one-seventh of their parenteral potency in agreement with the results obtained by Turner and Reineke (1946) on the utilization of synthetic thyroprotein by sheep. Explanations for this poor utilization of synthetic thyroproteins might be found in the large steric hindrance to enzyme action resulting from the high percentage of iodine or in the

³ Explanations for the fact that natural thyroid proteins consistently show more thyroxine-like activity than indicated by their L-thyroxine content have been summarized recently by Frieden and Winzler (1948) and by Hamilton, Albert, and Power (1948) in addition to earlier discussion in the texts mentioned in footnote 2.

elimination of the sensitivity of many peptide linkages to the cleaving action of the endopeptidases, chymotrypsin and pepsin, which require intact tyrosine units. Other gross structural changes possibly attributable to iodine cannot account for our observations because of the insensitivity of casein to denaturation and the fact that substrate denaturation frequently increases ease of proteolysis.

SUMMARY

The comparative oral thyroxine-like activity of physiological range doses of DL-thyroxine and natural and synthetic thyroproteins has been measured using the goiter prevention method. The following conclusions were drawn:

DL-thyroxine demonstrated half of its parenteral activity when administered orally as the soluble sodium salt.

Four different natural thyroid preparations assayed 70%, 71%, 72%, and 98% of their parenteral potency. This activity was greater than could be attributed to the L-thyroxine content of the test substances when compared with parenterally administered thyroxine.

Synthetic thyroproteins showed only one-seventh of their parenteral activity, indicating poor absorption from the small intestine.

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OCCURRENCE AND DETERMINATION OF CONJUGATED SULFATES OF ESTROGENS IN URINE FROM PREGNANT WOMEN

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THE ISOLATION of estriol glucuronide from pregnant women's urine (Cohen, Marrian, and Odell, 1936) and estrone sulfate from pregnant mares' urine (Schachter and Marrian, 1938) demonstrated the fact that the estrogens and their metabolites may be excreted as water-soluble esters of glucuronic or sulfuric acid. Estrogens, other than estriol, have been found in human pregnancy urine, but their conjugates have never been isolated. Cohen and Marrian (1936) recognized the importance of this problem and Marrian (1937) wondered whether the estrone in human urine will be found to be conjugated with glucuronic acid like estriol or with sulfuric acid, as it is in pregnant mares' urine. Butenandt and Hofstetter (1939), although never actually isolating estrone sulfate from human pregnancy urine, concluded on the basis of solvent fractionations as well as chromatographic studies, that estrone was excreted by the human as the sulfuric acid ester because the partitioning properties of the estrone fraction resembled those of synthetic estrone sulfate. Crepy (1946) incubated estrone, estradiol and estriol with liver slices and concluded that estradiol and estriol were conjugated for the most part, with glucuronic acid, whereas, estrone seemed to be conjugated with another substance resembling sulfuric acid.

The results of Peterson et al. (1938) and Venning et al. (1942) showed that the androgenic steroid hormones in human male urine may be conjugated with either glucuronic acid or sulfuric acid. Wilder Smith (1948) claimed indirect evidence for the excretion of synthetic estrogens conjugated with sulfuric as well as glucuronic acid.

The present paper concerns itself with studies to determine the proportion of estrogenic conjugates that occur as the sulfate ester in pregnant women's urine. Utilizing the specific phenolsulfatase present in extracts of *aspergillus oryzae* (Abbot, 1947; Cohen and Bates, 1949), the estrogens conjugated with sulfuric acid were hydrolyzed by Mylase P and became extractable from water with ether or toluene, whereas, the estrogens conjugated with glucuronic acid were

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not hydrolyzed by the enzyme and remained soluble in water. It was found that both the weak phenolic and the strong phenolic estrogens are excreted, in part, as conjugated sulfates.

MATERIALS AND METHODS

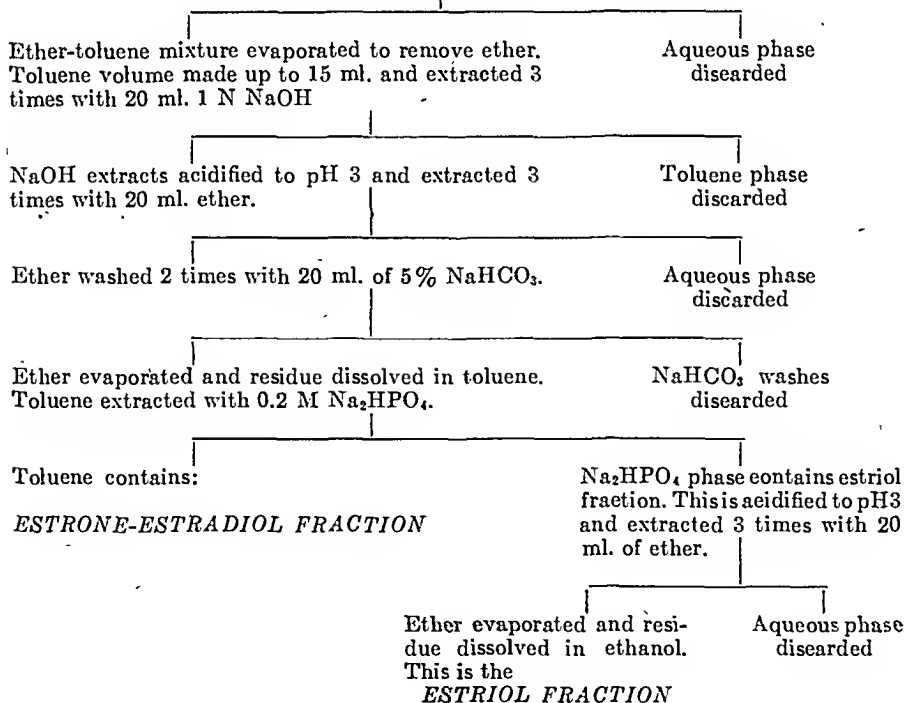
Urine from women who were in the 7th-9th month of pregnancy was used. When urine was used as substrate, the urine was collected under toluene and the toluene layer withdrawn to remove the free estrogens before digestion with Mylase P. In the cases where butanol extracts were used, the urine was collected under butanol, and then extracted with butanol. After the butanol was removed in vacuo, the residue was dissolved in water (pH 6-7) and the free estrogens were removed by extraction with ether, leaving a clear aqueous fraction which was used as substrate for enzyme and acid hydrolysis as well as for control tests.

Mylase P¹ was used as a source of phenolsulfatase. Enzyme digestions performed under a layer of toluene at 50°C. and pH 6.0 for a period of 24 hours, using 200 mg. of Mylase P per 100 ml. of urine or its equivalent. These conditions have been found to result in complete hydrolysis of estrogenic sulfates (Cohen and Bates, 1949). Controls for the enzyme digestion were prepared and processed exactly as was the enzyme digest except that no

Fig. 1

Procedure for Extraction of Estrogens

Enzyme digest or acid hydrolysate with toluene phase was extracted two times with 50 ml. of ethyl ether.



enzyme was added. Blank tubes containing enzyme alone gave negative tests for estrogens.

Acid hydrolysis of urine was performed, after the addition of 15 volumes per cent hydrochloric acid, C. P., by boiling for fifteen minutes under a layer of toluene. Acid hydrolysis of the butanol extract preparations was performed by acidifying the solutions to pH 0.8 and boiling them for fifteen minutes under a layer of toluene.

Extraction of enzyme digests and acid hydrolysates were carried out according to the procedure in Figure 1. The phenolic fraction was separated into an estriol fraction and an estrone-estradiol fraction, using 0.2 M. disodiumphosphate, as recommended by Friedgood et al. (1948). In one case (Table 1, D.) the fractions were separated using the chromatographic method of Stimmel (1946)

Estrogen determinations were made by the fluorimetric method of Bates and Cohen (1947). An estrone standard was used for the estrone-estradiol fraction and an estriol standard for the estriol fraction. In the case of the estriol fraction, confirmatory positive tests by a modified Kober method (Cohen and Bates, 1947) and negative tests by a modified Zimmerman method (Holtorff and Koch, 1940) were obtained. Because of the low estrogen content of the estrone-estradiol fraction, Kober and Zimmerman determinations were not made.

RESULTS

The data are shown in Table 1. The percentage of estrogen conjugated with sulfuric acid was calculated using the acid hydrolysis value as the value for the total estrogen content of each fraction and the difference between the enzyme and control values as the amount hydrolyzed by the enzyme. It can be seen that the amount released

TABLE 1. PERCENTAGE OF ESTROGENS CONJUGATED AS THE SULFATE IN HUMAN URINE OF LATE PREGNANCY AS ESTIMATED BY HYDROLYSIS WITH MYLASE P. THE FREE ESTROGENS WERE REMOVED PRIOR TO TREATMENT BELOW.

Patient	Pregnancy stage	Nature of substrate	Urine vol. equivalent	Method of hydrolysis	Estrone-estradiol as estrone	Estriol as estriol	Estrone-estradiol fraction	Estriol fraction
					micrograms	micrograms		
A	months 8	Urine	100	Enzyme Acid	70 150	400 2,000	46%	20%
B	7	Urine	500	Enzyme Control Acid	130 113 320	120 26 1,700	8%	6%
B	8	Butanol Extract	320	Enzyme Control Acid	26 7 57	45 8 540	38%	8%
C	9	Urine	120	Enzyme Control Acid	19 7 43	25 0 550	33%	5%
C	9	Butanol Extract	120	Enzyme Acid	23 110	100 950	21%	10%
C	9	Butanol Extract	160	Enzyme Acid	35 35	160 180	100%	89%
D	8	Butanol Extract	1,000	Enzyme Control Acid	45 2.5 50	52 0 120	84%	43%

by the enzyme digestion varies from 8 per cent to 100 per cent for the estrone-estradiol fraction while that portion of the estriol fraction hydrolyzed by the enzyme varied from 5 per cent to 89 per cent. In general, a greater proportion of the estrone-estradiol fraction, than of the estriol fraction, is conjugated with sulfuric acid. Presumably, the balance of the estrogen in all cases is conjugated with glucuronic acid.

DISCUSSION

The conjugation of estrogens in human urine, with both sulfuric and glucuronic acid, has been postulated (Cohen and Marrian, 1936) but no sulfate-conjugated estrogen has ever been isolated. This, no doubt, is due to the small amount of sulfate-conjugated estrogens present in human urine. However, this limitation is made less insurmountable with the development of fluorimetric analytical methods which permit quantitative estimation of as little as 0.5 micrograms of estrogen (Bates and Cohen, 1947; Jailer, 1947; Finkelstein et al., 1947). Utilizing such methods, together with specific enzyme hydrolysis, it is now possible to demonstrate the presence of such sulfate-conjugated estrogens in human urine. Of special interest is the finding that a large portion of the estriol in urine may sometimes be conjugated with sulfuric acid.

Since it has been shown that Mylase P contains no glucuronidase or general phenol esterase (Fishman, 1948) and since it also has been shown that Mylase P contains a phenolsulfatase (Abbott, 1947; Cohen and Bates, 1949) it is concluded that the urinary estrogens hydrolyzed by the enzyme mixture existed in the urine as a water-soluble sulfate. It may also be conjectured, that, inasmuch as the phenolsulfatase of *aspergillus oryzae* does not hydrolyze alcoholic sulfates (Fromageot, 1938), the point of conjugation of estriol with sulfuric acid must be at the 3 phenolic hydroxyl position in the estriol molecule, thus differing from the point of glucuronic acid conjugation, which has been shown by Marrian (1937) to be at the 16 or 17 alcoholic hydroxyl position.

SUMMARY

The occurrence of estrogens conjugated with sulfuric acid in pregnant women's urine was demonstrated by the use of an enzyme preparation containing phenolsulfatase.

The sulfate ester linkage must be with the phenolic hydroxyl group at position 3 since phenolsulfatase does not hydrolyze alcoholic sulfates.

Among seven different urine samples a variable proportion of from 5 per cent to 89 per cent of the estriol fraction and 8 per cent to 100 per cent of the estrone-estradiol fraction was found to be conjugated as the sulfate.

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PITUITARY AND ADRENO-CORTICAL RELATIONSHIPS TO LIVER REGENERATION AND NUCLEIC ACIDS

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WE HAVE found (1949) that thyroidectomy in young animals reduces the capacity for liver regeneration in rats after partial hepatectomy; and that administration of desiccated thyroid to both young (2 months old) and older (3 to 5 months old) animals increases the rate of regeneration. We here report experiments on the influence of the pituitary and the adrenals. The results indicate that hypophysectomy depresses liver regeneration, but that there is no evidence of any adrenal effect. Other results will be discussed in the text.

METHODS

Inbred rats of the Wistar strain (a few of the Sprague-Dawley) were used. Sex apparently had no influence on the results, and the results include data on both sexes. Partial hepatectomy was performed by removing the left lateral and median lobes. It has been amply established that this removes, for our purpose, with negligible error about 2/3 of the liver. The liver was allowed to regenerate for 96 hours, and the amount, which can be variously calculated, was determined by the following formula:

$$\% \text{ regeneration} = \frac{\text{wt. of liver at autopsy} - \frac{1}{2} \text{ amount removed}}{\text{amount removed}} \times 100.$$

This represents in per cent approximately the amount of removed liver replaced by new tissue.

All surviving animals were sacrificed 96 hours after partial hepatectomy, by which time the most rapid phase of regeneration has been completed. The animals were maintained on a standard diet of 25% casein, 50% sucrose, 20% Crisco, 5% salt mix (Phillips and Hart, 1935); and a vitamin supplement of Vipenta 0.5 ml/kilo. Hypophysectomy and adrenalectomy both affect the appetite; in both cases, therefore the normal animals were restricted to the average food intake of the group for which they acted as controls. The animals that were given adrenocortical extract were kept at the same dietary level as the adrenalectomized animals and their normal controls. The hypophysectomized animals and their controls were five weeks old at partial hepatectomy; the adrenal experimental group was two months old. Nucleic acids were determined by the method of Schmidt and Thannhauser (1945).

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RESULTS:

(1) *Pituitary*. The animals were hypophysectomized at age of 28 days, by Dr. Roy O. Greep, to whom we wish to express our obligation, and were lobectomized 5-7 days later. The data concerning this group and their controls are summarized in Table 1. The striking thing is the difference in the 96 hour regeneration. In the normal animals on a restricted diet this amounted to 76 per cent; in the hypophysectomized, 39 per cent. The "T" value was over 3, and the results statistically significant.

Franseen, Brues and Richards (1938) were of the opinion that the decreased liver regeneration of hypophysectomized animals was chiefly due to the diminished food intake, though Higgins and Ingle (1939) stated that "on the basis of total average animals, it is possible that the loss of pituitary gland may have had some inhibitory effect on the extent of regeneration." Their caution in interpretation in spite of the fact that the hypophysectomized rats actually did regenerate less than controls on the same diet, was due to doubts concerning the absorption and utilization of diets and differences in muscular activity. In so far as this might affect the relation of available nutritive material to the energetic needs, and hence the relative state of nutrition, this was not a factor in our experiments, since not only was the average amount eaten by the control and hypophysectomized animals the same, but their change in weight during the experiment was not significantly different. Unless the latter were coincidental, due to compensatory differences in hydration, which there is no reason to believe, we can say that the diet was not a factor in our experiments and that the influence of the pituitary on liver regeneration would appear to be established.

In the pre-lobectomized liver, hypophysectomy resulted in a fall in the ribonucleic acid (PNA) fraction, but no change in desoxyribonucleic acid (DNA), nitrogen, or water concentration. After four days' regeneration following lobectomy, there was what appeared to be a significant (though barely so) rise in the DNA concentration in hypophysectomized as compared with the control animals. This is of interest, because in the experiments cited above, we have found that thyroidectomy in young animals results not only in a decreased rate of liver regeneration, but also a fall in the nucleic acid concentration. It would seem therefore that the amount of nucleic acid mobilization and of regeneration do not necessarily go hand in hand. Also, so far as it goes, it tends to indicate that the pituitary influence on regeneration is not via the thyroid. This point, however, requires further investigation.

(2) *Adrenals*.

(a) *Adrenalectomy*. A group of animals were adrenalectomized and then partially hepatectomized 10 days later, when they were about two and a half months old. At first, none of our adrenalectomized

TABLE I. EFFECTS OF HYPOPHYSECTOMY

	Pre-lobectomized liver					Regenerated liver				
	Nucleic acids			Dry wt.		Nucleic acids			Dry wt.	
	PNA	DNA	Total	Wet wt.	Total N	PNA	DNA	Total	Wet wt.	Total N
Normal (14)*	mgm. P/100 gm. wet wt. 111 ± 3.0†	25 ± 2.4	138 ± 3.8	30 ± 0.4	3.24 ± .096	mgm. P/100 gm. wet wt. 114 ± 2.3	27 ± 2.3	141 ± 3.4	28 ± 0.5	3.04 ± .055
Hypophysectomized (26, 10)	98 ± 1.7	26 ± 1.8	124 ± 2.5	29 ± 0.5	3.13 ± .082	115 ± 2.8	38 ± 2.7	153 ± 3.9	28 ± 1.4	3.23 ± .138

* Numbers in parentheses refer to number of experimental animals. Where there are two numbers in parentheses, the second indicates the number that survived hepatectomy for regeneration studies.

† Standard Deviation of the Mean = $\sqrt{\frac{\sum d^2}{n(n-1)}}$.

TABLE II. EFFECTS OF ADRENALACTOMY AND ADRENOCORTICAL EXTRACT INJECTIONS

	Pre-lobectomized liver						Regenerated liver						
	Nucleic acids				Dry wt.	Total N	Nucleic acids				Dry wt.	Total N	Regenera- tion %
	PNA	DNA	Total	mgm. P/100 gm. wet wt.			PNA	DNA	Total	mgm. P/100 gm. wet wt.			
Kept at 20°-25° Normal	(10)*	102 ± 0.9†	21 ± 0.7	123 ± 1.1	29 ± 0.2	2.93 ± .081	110 ± 2.8	22 ± 0.4	132 ± 2.8	30 ± 0.6	2.55 ± .088	63 ± 6.4	
Kept at 31°	(12)	110 ± 0.4	21 ± 0.1	131 ± 0.4	29 ± 0.6	3.24 ± .042	111 ± 0.6	23 ± 0.6	134 ± 0.8	30 ± 0.7	3.23 ± .107	52 ± 3.4	
Kept at 20°-25° Adrenalectomized	(16)	103 ± 1.9	21 ± 0.9	124 ± 2.1	29 ± 0.4	2.95 ± .046	123 ± 2.3	28 ± 1.0	151 ± 2.5	26 ± 0.3	2.68 ± .014	56 ± 4.9	
Kept at 31°	(15)	108 ± 1.5	26 ± 0.6	134 ± 1.6	36 ± 0.3	3.74 ± .089	111 ± 1.2	26 ± 0.3	137 ± 1.2	34 ± 0.5	3.39 ± .116	46 ± 5.0	
5 rat units daily ACE (infected for 5 days)	(10)	97 ± 1.5	18 ± 0.7	115 ± 1.7	30 ± 0.3	2.70 ± .024	119 ± 3.4	21 ± 0.4	140 ± 3.4	29 ± 0.6	2.55 ± .055	56 ± 6.4	
16 rat units daily	(3)	104 ± 2.1	15 ± 0.0	119 ± 2.1	30 ± 0.0	2.96 ± .024	117 ± 1.4	25 ± 0.0	142 ± 1.4	27 ± 0.1	2.74 ± .170	49 ± 6.5	

* Numbers in parentheses refer to number of experiments.

† Standard Deviation of the mean = $\sqrt{\frac{\sum d^2}{n(n-1)}}$.

animals survived the removal of two-thirds of the liver, death occurring invariably within 24 hours. After some experimentation, however, we found that if 10 ml. of an isotonic NaCl solution was introduced into the peritoneal cavity at the time of hepatectomy, the great majority of the animals survived when given 1% NaCl in their drinking water. These were compared with a control group of the same age kept at the same food intake. The results are recorded in Table 2. They can be summarized by saying that adrenalectomy had no significant effect on the rate of regeneration, nor on the nitrogen or water content of the liver. There was, however, after adrenalectomy a greater concentration of nucleic acids in the regenerating liver than in the controls, apparently affecting both fractions. (The absolute amount was approximately the same). This is not observed in the liver before hepatectomy. Comparing this with the nucleic acid patterns described in the previous section, it is evident that no definite relationship exists between the regenerative activity of the liver under the different conditions considered, and the nucleic acid concentration.

Berman, Sylvester, Hay and Selye (1947) have recently reported that adrenalectomy decreases the amount of liver regeneration in the first 24 hours to an extent not accounted for by the decreased ability to mobilize fat. Our own results, for the 96 hour regeneration, as indicated, do not agree with this. Since the regeneration of the first 24 hours involves little or no mitosis, and is chiefly a matter of fat and water mobilization, possibly a distinction should be made between the regeneration of this period and that of later periods, since the liver events of the first day after lobectomy are in a sense largely a preparation for the cell regeneration that occurs later.

In view of the fact that a few adrenalectomized animals, in hot summer weather, showed a tendency to a lower regeneration than animals under more temperate conditions, we thought it might be possible that under such a stress the effect of adrenalectomy might be made manifest. Through the kindness of Dr. Eugene M. Landis, who made available to us a constant temperature room, we were enabled to maintain a group of adrenalectomized animals and their controls for ten days before, as well as for the four days after hepatectomy, at a temperature of 31°C. The results will be seen in Table 2. The adrenalectomized animals showed a smaller water content than their controls, and than both controls and adrenalectomized at moderate temperatures. Both groups at the high temperatures had a high nitrogen concentration, though little more than might be expected from the fall in water content. The regeneration was somewhat reduced in both groups. It is apparent that the stress of high temperature did not preferentially affect the adrenalectomized animals, except in the matter of the liver dehydration.

(b) *Injection of Adrenocortical Extract (ACE)*. Into a group of ten animals we have injected five units of ACE² in two divided doses daily on the day prior to hepatectomy and during the four days following. It will be seen in Table 2 that this was entirely without effect. To three more animals we administered fifteen units daily in the same manner. This, too had no appreciable effect.

From this evidence, and that obtained from adrenalectomy, we must conclude that the adrenal cortex exerts no influence on the regeneration of liver. It follows, of course, that the demonstrated influence of the pituitary is not via the adrenal cortex.

SUMMARY

We have investigated the influence of the pituitary and adrenals on liver regeneration following partial hepatectomy, and upon nucleic acid and total nitrogen concentration, the effect of diet and nutritional status being controlled.

Hypophysectomy results in a marked diminution in regeneration and a change in nucleic acid partition and concentration.

Adrenalectomy causes an increase in nucleic acid concentration in the regenerated liver; this is not reversed when ACE is injected. However, neither adrenalectomy nor the administration of adrenal-cortex extract has any effect on the amount of liver regeneration.

The control exercised by the pituitary on the regeneration is hence not exerted through the adrenals; the evidence suggests that it is also not mediated through the thyroid.

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² We wish to express our obligation to Dr. Dwight J. Ingle, and Dr. Marvin H. Kinzinga, of the Upjohn Company, for the adrenal-cortex extract used in this work.

SEROLOGICAL STUDIES ON CRYSTALLINE ADRENOCORTICOTROPHIC HORMONE: THE PRODUCTION OF ANTI- ADRENOCORTICOTROPHIN¹

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CURRENT interest in the adrenocorticotrophic hormone has continued to expand at a rapid rate, and a vast quantity of data concerning the physiology and metabolism of this hormone and its influence upon the adrenal gland, has been accumulated. In view of this widespread use of the adrenocorticotrophic hormone, both in clinical and animal experimentation, the problem of the formation of a substance antagonistic to the action of this hormone is a pertinent one.

It is a well known fact that certain hormones, when administered parenterally over a long period of time, stimulate the production of substances which have been called "antihormones." These substances have the property of inhibiting the action of any further injected hormone. The phenomenon of antihormone formation and action was discovered by Collip (1934; 1935) during the course of his work with the pituitary-trophic hormones; it was found that sera of refractory animals (those which did not respond to injections of pituitary gonadotrophic hormone) protected immature female rats against the normal action of this hormone. Collip and Anderson (1934) also demonstrated a similar result with the thyrotrophic hormone.

Although the mechanism of hormone-antihormone action is still a matter of controversy, the results of various investigators indicate that antihormones behave, for the most part, as antibodies; and that, therefore, the term *antihormone* may be defined immunologically as *an antibody produced in response to the antigen, hormone* (Thompson, 1946). The controversy, which is still in existence, is the result of the fact that relatively few of the hormones have been isolated in pure form, and of those which have been crystallized, few are available in large enough quantities for experimental purposes. As a result, those investigators who do not regard *antihormones* and *antibodies* to

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hormones to be synonymous, postulate that any *in vitro* antibodies which can be demonstrated in the sera of refractory animals are non-specific, in that they are due to impurities in the antigen preparations.

Of the pituitary hormones, the gonadotrophic, thyrotrophic and adrenocorticotrophic demonstrate the greatest degree of antigenicity (Thompson, 1946). The sera of normal, untreated individuals have been reported to contain antibodies to the gonadotrophic hormone, although this phenomenon occurs only in rare instances (Collip, 1935; and Collip and Anderson, 1939). Chow (1942) working with "pure" hog-pituitary gonadotrophic hormone, found this material to be antigenic, the homologous antiserum being both species- and hormone-specific. Equine gonadotrophic hormone (pregnant mares' serum), which is used extensively clinically, has also been shown to be antigenic (Rowlands and Spence, 1939; Jailer and Leathem, 1940). Allergic reactions have also been elicited with this preparation (Fluhmann, 1940; Erving, Sears and Rock, 1940; and Bickers, 1941).

Using 4 commercial preparations of gonadotrophic hormone, the author has previously demonstrated the formation of precipitins to each of these substances (Chase, 1945). These antibodies were shown to have identical properties with other immunologically defined antibodies. Suitable serological methods for demonstrating the precipitating antibodies were developed. These methods utilized collodion particles to increase the surface of the antigen, since the gonadotrophic hormone is thought to be a relatively small particle of glycoprotein. It was also shown in this series of experiments that the production of serologically demonstrable antibodies to the gonadotrophic hormone was accompanied by an increase in biological activity and *vice versa*. Electrophoretic studies in the Tiselius apparatus revealed an increase in the gamma-globulin portion of the blood serum of injected animals, which could be correlated with the increase in precipitating antibodies as well as with the increase in biological activity. The mechanically separated gamma-globulin exhibited three times the precipitating and biological activity of the whole serum. Since the nitrogen content of the gamma-globulin portion was shown to be much less than was that of the whole serum (Micro-Kjeldahl determinations), it was concluded that the antibody was contained in the gamma-globulin portion of the blood serum.

It has recently been shown that, in rabbits, the route of injection has a marked influence upon the rapidity with which inhibitory substances for the gonadotrophic hormone are formed (Leathem, 1947). Antihormones were formed more readily when the subcutaneous route of injection was used. These results have been confirmed by the author, using Gonadin, a commercial pregnant mares' serum preparation (Chase, unpublished results). In immunological circles, it is an

established fact that the antibody response to a given antigen is influenced markedly by the route of injection of the latter.

Again, there have been reports of the appearance spontaneously of the antithyrotrophic factor in the sera of untreated individuals (Simon, 1936; Picado and Rotter, 1936). However, it has been impossible to demonstrate biologically active antagonists in patients with Graves' disease until treatment has been instigated (Spence and Scowen, 1934; Picado and Rotter, 1936; and Scowen and Spence, 1936). The titer of anti-thyrotrophin has been reported to be high in a case of Cushing's syndrome (Eisenhardt and Thompson, 1939). Finally, reports of the development of antithyrotrophic factors in the sera of both man and animal do exist, and the results are similar to those obtained with the antigonadotrophic factor (Spence and Witts, 1939; Collip and Anderson, 1934; Okkels, 1937; Cope, 1938; and Cutting, Robson and Emerson, 1939).

The present study was undertaken to determine whether or not purified adrenocorticotrophic hormone would elicit antihormone formation after injection into suitable experimental animals. Further, in substantiation of the hypothesis that the reaction of a hormone and its specific antagonist is a true immunological phenomenon, it was sought to produce an *in vitro* reaction between the crystalline adrenocorticotrophic hormone and its homologous antiserum.

MATERIALS AND METHODS

Animals. Normal mice of both sexes (NHO strain, Strong; and Swiss white), 60–80 days of age, were used for the production of antibodies to the adrenocorticotrophic hormone. Twenty-one-day-old, white, male rats were used to study the biological activity of the antiserum against the adrenocorticotrophic hormone. Food and water were provided *ad libitum*. Both mice and rats were fed Purina Fox Chow. All animals were kept in the laboratory for at least one week prior to their use.

Hormone preparations. The adrenocorticotrophic hormone preparation used was the one described by Sayers, White and Long (1943). The preparation fulfilled two criteria for protein purity: it behaved as a single component in the Tiselius apparatus and in the ultracentrifuge. The preparation exhibited none of the following pituitary hormonal activities in biological assay determinations: growth, gonadotrophic, thyrotrophic or lactogenic.

Crude Prolactin was used as a control material in the biological assay experiments. From previous studies, this material was known to contain assayable quantities of adrenocorticotrophic hormone.

Antisera. Blood was obtained from all mice by cardiac puncture. The bloods were pooled, allowed to clot in the refrigerator and the sera separated by centrifugation. All sera were kept in the frozen state until needed.

In certain of the *in vivo* experiments, antigonadotrophic serum from a dog "Alice" was used (Thompson and Cushing, 1937). The dog was injected for many months with a crude sheep pituitary preparation, and its serum contained antagonists to the pituitary gonadotrophic hormone as well as to other pituitary hormones. The dog serum was maintained in the lyophilized state.

When needed, it was standardized so that 0.1 ml. of solution contained 5 mg. of lyophilized preparation.

Collodion particles. Collodion particles were used in several of the serological tests, to increase the surface of the antigen (adrenocorticotrophic hormone), and thus produce particles within the range of visibility. These were prepared according to the methods of Cannon and Marshall (1940) with a few modifications.

When the collodion particles were to be coated with antigen, a suspension dense enough to be milky, was added to about twice its volume of adrenocorticotrophic hormone, and the mixture was allowed to remain in the refrigerator over a period of 48 hours. If the particles settled while in contact with the antigen, they were readily resuspended by shaking. The coated particles were then washed by centrifugation and decantation and were finally resuspended in a small amount of triply distilled water. The pH was adjusted to 6.0 with 0.01N HCl and a buffer at this pH was added, in the proportions of one part buffer to 10 parts suspension. The buffer solution was prepared by mixing 12.5 ml. 0.2 M sodium acetate and 0.3 ml. of 0.2 M acetic acid and diluting to 250 ml. with triply distilled water. The buffer tended to stabilize the particles so that the adsorbed suspension could be kept in the refrigerator for longer periods of time.

When the adrenocorticotrophic hormone-collodion particle complex was needed as the antigen in the serological tests, a small quantity of the stock mixture was diluted with physiological saline until it corresponded with tube #5 of the MacFarland nephelometric standard. The antigen-collodion particle complex was then added in constant amounts to varying dilutions of the antiserum.

Protocol of immunization. The first group of mice received gradually increasing daily doses of pure adrenocorticotrophic hormone intraperitoneally over a period of 25 days. A total of 50 mg. of the hormone was administered to each mouse. Each dose of hormone was dissolved in 0.3 ml. of 0.85 per cent saline. Control mice received injections of saline alone. The mice were bled one week after the first injection, one day after the last injection and again, 10 days after the last injection.

The second group of mice were treated in exactly the same manner except that the injections were given subcutaneously. Ten control mice received injections of 0.3 ml. 0.85 per cent saline.

Biological Assay Methods. In studying the effect of pure adrenocorticotrophic hormone on the adrenal weights of 21-day-old, male rats, 0.05 mg. of hormone in 0.1 ml. 0.85 per cent saline were injected subcutaneously three times a day for three days. During this three-day period, the rats were weighed daily. Sixteen hours after the last injection, the rats were again weighed, then killed with illuminating gas. The adrenals were removed immediately and weighed on a torsion balance. The adrenal weights were finally expressed as mg./100 gm. initial body weight.

In the experiments where antiserum was injected, this was administered subcutaneously in 0.05 ml. quantities twice a day for 3 days so that a total of 3.0 ml. in all were injected.

When both the adrenocorticotrophic hormone and the antiserum were used, hormone injections were begun one day after serum injections and, therefore, lasted one day longer.

Serological Methods. When needed, the antiserum was allowed to thaw at room temperature. Progressive dilutions were added by the doubling dilution technique (1:2 to 1:2560) to a series of serological test tubes. All dilutions were made with 0.85 per cent saline prepared with triply distilled water, and each tube contained 0.5 ml. diluted serum. Each test was done in duplicate.

When adrenocorticotrophic hormone-collodion particle complex was used as the antigen, 0.5 ml. of a suspension corresponding to tube # 5 of the MacFarland nephelometric standard was added to each tube of diluted serum. Controls included 1:10 antiserum alone, 1:10 antiserum plus unadsorbed collodion particles, and hormone-collodion particle complex alone. Routinely the racks of tubes were incubated overnight in a water bath at 37 deg. C. After the incubation period, the tubes were centrifuged in an angle centrifuge at approximately 1200 r.p.m. for 5 minutes, and then "flipped." Tubes in which a precipitate occurred which adhered to the bottom of the tube and did not resuspend even after vigorous shaking, were interpreted as positive.

In several cases, precipitin tests were set up in which the adrenocorticotrophic hormone alone (unadsorbed) was used as antigen. Antiserum dilutions were made in the same manner as when the hormone-collodion particle complex was used as the antigen. To each tube of diluted antiserum, 0.5 ml. of 0.85 per cent saline containing 0.025 mg. adrenocorticotrophic hormone were added. The racks of tubes were incubated at room temperature for 2 hours. Tubes in which there occurred a macroscopically visible precipitate were recorded as positive.

EXPERIMENTAL AND RESULTS

Biological Assay Experiments. Since the adrenocorticotrophic hormone used in these experiments was a pure preparation, it was interesting to note whether or not a serum from mice, treated daily with this hormone for 25 days, would inhibit the normal action of the latter. The results of this experiment are recorded in Table 1. It may be seen from this table that the mouse anti-adrenocorticotrophic serum exerted a pronounced inhibitory effect on the normal action of the adrenocorticotrophic hormone. When a total of 0.45 mg. of hormone was injected into each of 5 rats, an average adrenal weight of 77.2 mg./100 gm. body weight was obtained. When both hormone and serum from mice, treated for one week with adrenocorticotrophic hormone, were injected into a group of 10 rats, the average adrenal weight was 51.3 mg./100 gm. body weight. With sera obtained 1 day after the series of injections had been completed, the inhibitory effect was even greater (average adrenal weight = 41.2 mg./100 gm. body weight). Sera obtained 10 days after the series of injections had been completed produced the most marked inhibitory effect on the normal action of the adrenocorticotrophic hormone (average adrenal weight = 36.3 mg./100 gm. body weight).

It may also be seen from the results in Table 1 that crude Prolac-

tin exerts a definite adrenocorticotrophic action, when injected into 21-day-old, male rats, in that the adrenal weights in these animals were greater than those of the normal controls.

When dog antigenadotrophic serum was used, the action of the adrenocorticotrophic hormone on the adrenals was inhibited although this inhibition was not as marked as when mouse antiserum was injected. The action of the crude Prolactin was also inhibited both when the dog antigenadotrophic serum and the specific mouse anti-

TABLE 1. BIOLOGICAL ACTIVITY OF MOUSE ANTISERA SPECIFIC FOR ADRENOCORTICOTROPHIC HORMONE

Number of rats	Treatment	Average adrenal wts./100 mg. body wt.
5	ACTH ¹	77.2 mg.
5	ACTH + Normal Mouse Serum	76.9 mg.
4	Crude Prolactin ²	59.3 mg.
10	ACTH + Antiserum I ³	51.3 mg.
6	ACTH + Antiserum II ⁴	41.2 mg.
6	ACTH + Antiserum III ⁵	36.3 mg.
6	Crude Prolactin + Antiserum III	44.0 mg.
6	Crude Prolactin + dog (Alice) antigenadotrophic Serum ⁶	50.9 mg.
8	ACTH + dog (Alice) Antigonadotrophic Serum	41.6 mg.
6	Pooled normal Mouse Sera	35.5 mg.
6	—	35.1 mg.
6	Antiserum III	27.5 mg.
6	Dog (Alice) Antigonadotrophic Serum	34.6 mg.

¹ Pure adrenocorticotrophic hormone. A total of 0.45 mg. in 0.9 ml. 0.85 per cent saline was injected, intraperitoneally.

² A total of 9 mg. in 0.9 ml. saline was injected intraperitoneally.

³ Antiserum I was obtained from mice one week after the first injection of ACTH.

⁴ Antiserum II was obtained one day after the series of injections had been completed.

⁵ Antiserum III was obtained 10 days after the series of injections had been completed.

⁶ A total of 30 mg. in 6.0 ml. saline was injected subcutaneously.

adrenocorticotrophic serum were used; and again the inhibitory effect was greater with the latter.

Control animals included one group in which no injections were given, and a second in which normal mouse serum was used. Results were comparable in both control groups. When the specific mouse antiserum was injected without the stimulating hormone, the adrenal weights were slightly less (27.5 mg./100 gm. body weight) than those of the control groups (35.5 mg./100 gm. body weight; and 35.1 mg./100 gm. body weight), although the difference was not great enough to draw any conclusions as to the effect of the antiserum on the intact adrenal.

To all intents and purposes, the results of this experiment indicate that it is possible to produce in mice a serum which exerts a profound antagonism against the normal action of the adrenocorticotrophic hormone in its pure state. The titer of antagonistic factor in-

creases during the course of immunization and reaches a peak 10 days after the series of injections has been completed. Serum obtained at this time, and injected into the 21-day-old, male rats has been shown to inhibit completely the effect of the adrenocorticotrophic hormone in increasing adrenal weight.

Serological Experiments. As has been previously stated, definite proof of the nature of antihormones has not yet been offered because a pure protein hormonal antigen has never been used for the production of these antagonistic substances. Current opinion, however, favors the theory that antihormones belong to the class of substances called antibodies (Thompson, 1946). The results of the above *in vivo* experiments indicate that it is possible to produce in animals a substance, present in the serum, which has the biological effect of

TABLE 2. AGGLUTININ TITERS OF ADRENOCORTICOTROPHIC HORMONE-COLLIDION PARTICLE COMPLEX AND POOLED MOUSE ANTI-ADRENOCORTICOTROPHIC SERA

Number of mice	Treatment	Route of injection	Agglutinin titers, serum obtained			
			Pre-injection	1 week after 1st injection	1 day after last injection	10 days after last injection
20	ACTH ¹	Intraperitoneal	0	1:20	1:80	1:320
10	Controls—0.3 ml. 0.85% saline daily	Intraperitoneal	0	0	0	0
20	ACTH	Subcutaneous	0	1:40	1:160	1:320
10	Controls—0.3 ml. 0.85% saline daily	Subcutaneous	0	0	0	0

¹ Adrenocorticotrophic hormone. Each mouse received a total of 50 mg. in gradually increasing daily doses over a period of 25 days.

neutralizing the normal action of the pituitary adrenocorticotrophic hormone. Since the hormone used in these studies has been shown to be a single protein, it may be assumed that the antiserum is directed against the hormonal antigen and not against any contaminating protein antigen. Although the inhibition experiments may be likened to the passive immunization studies, so well known to all immunologists, it was sought to produce an *in vitro* reaction between the antigen, adrenocorticotrophic hormone, and the antibody, anti-adrenocorticotrophic factor, contained in the sera of mice which had received a prolonged course of injection of the antigen.

Two varieties of serological tests were employed although, in essence, their nature was identical. The first utilized collodion particles upon which the hormonal antigen had been adsorbed. Since the antigen is normally a soluble one, this process produced a large particle

which, when aggregated, was macroscopically visible. This type of test will hereafter be referred to as an *agglutination* test.

The results of the agglutination test are summarized in Table 2. It may clearly be seen from the results that, in the test tube, a macroscopically visible reaction occurs between the adrenocorticotrophic hormone-collodion particle complex and the specific mouse antiserum. Serum obtained before the course of injections had been initiated, failed to produce this reaction. The maximum titer (1:320) was obtained with the serum taken 10 days after the series of injections had been completed. Antibodies to the adrenocorticotrophic hormone were formed more readily and more rapidly when the subcutaneous route of administration was used. These results are in accord with those obtained in the *in vivo* experiments.

TABLE 3. PRECIPITIN TITERS OF ADRENOCORTICOTROPHIC HORMONE AND HOMOLOGOUS MOUSE ANTISERA

Number of mice	Treatment	Route of injection	Precipitin titers, serum obtained			
			Pre-injection	1 week after 1st injection	1 day after last injection	10 days after last injection
20	ACTH ¹	Intraperitoneal	0	1:20	1:80	1:320
10	Controls 0.3 ml. 0.85% saline	Intraperitoneal	0	0	0	0
20	ACTH	Subcutaneous	0	1:40	1:160	1:640
10	Controls 0.3 ml. 0.85% saline	Subcutaneous	0	0	0	0

¹ Adrenocorticotrophic hormone. Each mouse received a total of 50 mg. in gradually increasing daily doses over a period of 25 days.

Although collodion particles are considered to be an inert material, the possibility exists that they may exert some influence on the reaction between the adrenocorticotrophic hormone and its homologous antiserum. Therefore, a precipitin test was set up in which the antigen was the soluble hormonal protein. The results of this experiment are shown in Table 3. The maximum titer in the precipitin test was 1:640. This titer occurred with the serum obtained 10 days after the series of injections had been completed. As was the case with the agglutination test, precipitating antibodies appeared more rapidly when the subcutaneous route of injection was used in the production of the antisera.

Finally, an experiment was devised in which the supernatant layers from tubes containing visible precipitates were tested for biological activity. The supernatant layer was carefully removed from each tube after centrifugation, and was injected intraperitoneally into 21-

day-old, male rats. Each rat received 0.05 ml. 3 times a day for 3 days. Sixteen hours after the last injection, the rats were killed with illuminating gas, and their adrenals removed and weighed on a torsion balance. The results are tabulated in Table 4.

The results of this experiment confirm the results of the biological assay experiments. When normal mouse serum is combined with 0.025 mg. of adrenocorticotrophic hormone, no visible serological reaction occurs. Since there is no neutralization of the hormone action, approximately a twofold increase in adrenal weight is produced when

TABLE 4. EFFECT OF SUPERNATANT LAYERS FROM PRECIPITIN TESTS ON THE ADRENAL WEIGHTS OF 21-DAY-OLD, MALE RATS

Supernate Injected	Adrenal wt./100 gm. body wt. Rat injected with dilution								
	1:2	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280
0.025 mg. ACTH ¹ +Pooled Mouse Sera (Pre-injection)	mg. 61.2	mg. 60.0	mg. 58.4	mg. 60.9	mg. 59.8	mg. 61.0	mg. 62.4	mg. 61.1	mg. 61.8
0.024 mg. ACTH +Pooled Mouse Sera Obtained one Week after 1st Injection	29.5	31.0	30.8	31.5	29.9	36.4	47.0	54.3	59.8
0.025 mg. ACTH +Pooled Mouse Sera Obtained one Day after Last Injection	27.4	28.0	29.2	27.6	29.9	28.5	30.1	35.9	41.4
0.025 mg. ACTH +Pooled Mouse Sera Obtained 10 Days after course of Injections had been Completed	23.9	25.2	27.8	26.2	24.9	28.0	29.7	28.8	30.6
Control—Injected with Pooled Mouse Sera Obtained 10 Days After Completion of Series of Injections	20.9	20.0	22.3	22.0	25.7	25.4	28.6	28.0	27.2
Control—Saline	32.4	35.7	33.9	—	—	—	—	—	—

¹ Pure adrenocorticotrophic hormone.

the mixture is injected intraperitoneally into 21-day-old, male rats. However, when pooled mouse sera, obtained one week after the first injection of adrenocorticotrophic hormone had been given, was titrated against the hormone in the precipitin test, a titer of 1:40 was obtained. After the precipitate had been removed by centrifugation, the supernates of the 1:2, 1:10, 1:20, 1:40, 1:80 and 1:160 dilutions, produced no increase in adrenal weights of rats injected with the varying serum dilutions. In the dilutions beyond 1:160, the effect of the adrenocorticotrophic hormone was elicited. Sera obtained after the series of injections had been completed, produced comparable results both in the serological tests and in the biological assay experiments, in which the supernates from the precipitin tests were used.

It is interesting to note that, when varying dilutions of serum obtained 10 days after the series of injections of adrenocorticotrophic hormone had been completed, were injected into 21-day-old, male rats, there was approximately a 30 per cent decrease in adrenal weights over the saline controls. The results recorded in Table 1 suggested that the specific antiserum for the adrenocorticotrophic hormone may exert a direct influence on the intact adrenal glands of the 21-day-old, male rat. The results of the present experiment confirm these suggestions.

DISCUSSION

The data on the production of anti-adrenocorticotrophic sera is scarce. Thompson and Cushing (1934) mention the occurrence of refractoriness to the adrenocorticotrophic hormone in animals. These workers attempted to reproduce the Cushing syndrome in a puppy by injecting large doses of crude, ovine pituitary extract over a period of 4 months. The attempt failed, the researchers attributing their lack of success to the formation of anti-adrenocorticotrophic factor, after constant and prolonged injection of the pituitary extracts. Since the extracts used in these experiments were crude, however, no definite conclusions could be drawn as to the formation and nature of the antiserum.

The data presented in the present report substantiate previous suggestions of anti-adrenocorticotrophic formation after continuous parenteral administration of the protein hormone. The sera of mice, injected with pure adrenocorticotrophic hormone over a period of 25 days, gradually developed the ability to neutralize the action of the latter upon the intact adrenals of 21-day-old, male rats. A maximum titer of inhibition was obtained with sera taken 10 days after the course of injections of adrenocorticotrophic hormone had been completed.

Since the preparation of adrenocorticotrophic hormone used in these experiments was a pure protein, as shown electrophoretically and in the ultracentrifuge, it was possible to investigate the nature of the reaction between a hormone and its homologous antihormone. The results of serological studies, using the pure adrenocorticotrophic hormone as antigen, and pooled sera of mice, injected daily with this hormone over a period of 25 days, as a source of antibody, demonstrate conclusively that the reaction of the adrenocorticotrophic hormone and its respective antihormone is an immunological phenomenon and may be likened to any other reaction between an antigen and its homologous antibody. It is interesting to note that a prozone phenomenon occurred regularly in the precipitin test. Since this immunological phenomenon occurs commonly with many immunologically defined antigens and their homologous antisera, its occurrence with the hormonal antigen and the anti-adrenocorticotrophic sera may be interpreted as an indication of the immunological nature of the reaction between a hormone and its respective antihormone.

In the final experiment of this series, an attempt was made to correlate the biological activity of the specific antiserum, obtained by continuous injection of adrenocorticotrophic hormone into mice, with the serological activity. For this purpose, the precipitates formed in the test tube were removed by centrifugation, and the supernatant layers were tested for their biological activity. There was a definite correlation between biological and serological activities, as shown

from the results of this experiment. In serum dilutions, where proportions of antigen (adrenocorticotrophic hormone) and antibody (pooled mouse antisera) were optimal, the supernates showed no demonstrable effect on the adrenal weights of 21-day-old, male rats. When an excess of antigen was present in the supernate, there were increases in the adrenal weight of rats proportional to the quantity of hormone present. Under opposite conditions (when an excess of antibody was present), the size of the intact adrenals of 21-day-old rats was decreased proportionally.

The results obtained in this experiment indicate that the precipitate, formed in the serological tests, consists of a combination of hormone and antihormone and that, under conditions of optimal proportions, the precipitate represented the neutralized complex. As has been stated, the supernatant layers, in such cases, showed neither hormonal nor antihormonal activity when used in the biological assay experiments.

The experimental evidence presented in this paper offers data which support any previous hypotheses that antagonistic substances are formed in the sera of animals treated continuously with adrenocorticotrophic hormone. Since the hormone used was a pure one, it may be concluded that the antagonistic substance must be specific for the adrenocorticotrophic hormone, and not the result of stimulation by an unrelated contaminating adjunct. Further, the identity of the hormone-antihormone reaction with a true immunological phenomenon, in which a reaction takes place between an antigen and its homologous antibody, has been established. The serological activity of the antibody specific for the adrenocorticotrophic hormone has been correlated with the biological activity.

SUMMARY

A substance antagonistic to the normal action of the adrenocorticotrophic hormone has been demonstrated in the sera of mice treated by increasing daily doses of pure adrenocorticotrophic hormone over a period of 25 days. These sera were titrated in biological assay experiments against standard doses of the hormone in question, and were shown to prevent the increase in adrenal weights normally brought about by the adrenocorticotrophic hormone.

The serological activity of the antisera was demonstrated in both agglutination tests, in which collodion particles were used to increase the surface of the hormonal antigen, and in precipitin tests. Pure adrenocorticotrophic hormone was used as the antigen and the above described sera as the source of antibody. Maximum serological activity was obtained 10 days after the course of injections had been completed. The serological activity of the antiserum could be correlated with its biological activity.

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The author is indebted to Dr. Abraham White for making the various hormone preparations available, and for his invaluable suggestions during the course of these experiments.

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NOTES AND COMMENTS

THE LEVEL OF PLASMA PSEUDO-CHOLINESTERASE IN HYPOPHYSECTOMIZED AND HYPOPHYSECTOMIZED THYROXINE-TREATED RATS¹

It has been shown previously (Hawkins, Mendel and Nishikawara, 1948; Hawkins, Nishikawara and Mendel, 1948) that a depression in the level of thyroxine induced by thyroidectomy or by treatment with thiouracil results in a highly significant elevation in the activity of pseudo-cholinesterase in the plasma of male rats and, conversely, that an elevation in the level of thyroxine leads to a depression in the activity of this enzyme which is also highly significant. The level of true cholinesterase, on the other hand, is not affected by any of the above procedures.

This investigation has now been extended to include an evaluation of the effect of hypophysectomy on the inverse relationship between thyroxine level and plasma pseudo-cholinesterase activity in order to determine whether the hypophysis is an essential link in the reaction whereby the above relationship is established. The results of these experiments are to be reported here.

EXPERIMENTAL PROCEDURE

Adult male Wistar rats (200–250 g.) were divided into three groups, the members of two of which were hypophysectomized by the parapharyngeal approach. Functional hypophysectomy in all cases was established by histological evidence of adrenal cortical atrophy when the animals were sacrificed.² The rats were maintained on a moist diet of bread, milk and ground meat, with a water ration containing 10% sucrose. They were given daily injections of glucose and were housed in a constant temperature room at 28°C throughout the experimental period. Starting three weeks after operation, the survivors in the third group received injections of thyroxine³ (0.25 mg./100 g. body weight) every second day for a period of two weeks. Five weeks after the initiation of the experiment all animals were killed by exsanguination through the carotid artery under light ether anaesthesia. Oxalated blood samples were obtained and the plasma was used for estimation of pseudo-cholinesterase activity.

Enzymatic determinations were carried out manometrically in a medium of 0.025M NaHCO₃ saturated with 5% Co₂ (pH 7.4) with benzoylcholine (0.006M) serving as substrate (Mendel, Mundell and Rudney, 1943).

RESULTS

As the results in the accompanying table show, the pseudo-cholinesterase level in the plasma of hypophysectomized rats is elevated significantly above

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² We are indebted to Dr. W. S. Hartroft of this department for the histological examination of the adrenal glands.

³ Roche Organon (Synthetic Thyroxine).

TABLE 1. THE EFFECT OF THYROXINE ADMINISTRATION ON THE LEVEL OF PSEUDO-CHOLINESTERASE IN THE PLASMA OF HYPOPHYSECTOMIZED MALE RATS*

Group	No. of animals	Plasma Pseudo-ChE Activity	t	P
Normal	10	26.6 ± 2.7†	—	—
Hypophysectomized	6	51.3 ± 6.6	4.1†	1.9 × 10 ⁻⁴
Hypophysectomized, thyroxine-treated	8	15.7 ± 3.4	5.9§	3.1 × 10 ⁻⁷

* Activity is expressed as cu. mm. CO₂ evolved in 15 min. by 1 ml. plasma.

† ± represents standard deviation of the mean.

‡ 't' and 'P' values are in relation to values obtained for normal animals.

§ 't' and 'P' values are in relation to values obtained for hypophysectomized animals.

that found in unoperated control animals. A comparison of the enzyme levels found in the hypophysectomized, thyroxine-treated group with those observed in the hypophysectomized animals reveals that thyroxine is still capable of exerting a depressing action on the level of plasma pseudo-cholinesterase, in spite of the absence of the hypophysis. The hypophysis is therefore not an essential link in the reaction whereby the inverse relationship between the level of thyroid function and plasma pseudo-cholinesterase activity is established.

SUMMARY

Hypophysectomy leads to an elevation in the level of plasma pseudo-cholinesterase in male rats. This elevation is statistically significant.

Treatment of hypophysectomized rats with thyroxine results in a significant depression in the level of pseudo-cholinesterase in the plasma.

The hypophysis is therefore not an essential link in the reaction whereby the inverse relationship between the level of thyroid function and plasma pseudo-cholinesterase activity is established.

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ASSOCIATION NOTICE

THE SCHERING FELLOWSHIP FOR 1949 IS GIVEN TO DOCTOR D. LAWRENCE WILSON

Doctor D. Lawrence Wilson was selected as the first recipient of the Schering Fellowship. Doctor Wilson will work under the direction of Doctor George Thorn in the field of metabolic and endocrine diseases.

Doctor Wilson received the degree of Doctor of Medicine at Queen's University in 1944. Following his internship he served for eighteen months as Regimental Medical Officer in the R.C.A.M.C. He then received a Medical Research Fellowship for two years from the National Research Council of Canada, working during this period in the Department of Biochemistry of the University of Toronto. He fulfilled as Research Fellow the requirements for the degree of Master of Arts which he received in June 1948.

THE SQUIBB AWARD FOR 1949 IS GIVEN TO DOCTOR HERBERT M. EVANS

About thirty years ago Professor Herbert M. Evans began his studies on the Physiology of Reproduction. Some of this earlier work was published with J. A. Long in a monograph, "Oestrous Cycle of the Rat," a contribution which was of great importance in the investigation and subsequent isolation of one of the ovarian hormones. During the following three decades many significant contributions to Endocrinology were made by Evans and his associates, the most important perhaps being the discovery of the growth hormone and its subsequent purification and isolation.

Other important aspects of endocrinology have been studied by Evans and his associates in the University of California: (1) the corticotrophic hormones of the hypophysis; (2) the gonadotrophic hormones of the hypophysis, pregnant mare serum, and of the chorion; (3) the impairment of the vaginal cycle due to Vitamin A deficiency; (4) the recognition of the importance in the rat of a nutritional factor, X, for pregnancy and subsequently, the isolation and characterization of this factor, Vitamin E.

Doctor Evans was born in California in 1882. He studied medicine at Johns Hopkins, receiving the M.D. degree in 1908. He remained on the faculty of that institution until 1915 when he was called to the Chair of Anatomy at the University of California. In 1930 he was made Herzstein Professor of Biology and Director of the Institute of Experimental Biology.

THE AYERST, McKENNA AND HARRISON FELLOWSHIP FOR 1949 IS GIVEN TO DOCTOR ERNEST M. BROWN, JR.

Doctor Ernest M. Brown, Jr. was named to receive the Ayerst, McKenna and Harrison Fellowship for 1949. He was born in 1919 and received the degrees of Bachelor of Arts from West Virginia University in 1941 and of Doctor of Medicine from the University of Pennsylvania in 1944. He served

as intern and junior resident at the University of Pennsylvania Hospital 1944-46. Since that time until April 1948 he was a member of the Army Medical Corps. Doctor Brown will work at the George S. Cox Medical Research Institute with Doctor F. D. W. Lukens on lesions of the islands of Langerhans produced by intravascular infusion of glucose, continuing his studies begun under this Fellowship in 1948.

THE CIBA AWARD FOR 1949 IS GIVEN TO
DOCTOR GEORGE SAYERS

Doctor Sayers developed a new and sensitive method for the assay of the adrenocorticotrophic hormone of the anterior pituitary gland. He found that the ascorbic acid and cholesterol content of the adrenal glands varies inversely with the amount of ACTH administered to the test animal. Having established the method, he applied it to the problem of pituitary: adrenal relationships. The interrelation of the pituitary and the adrenal cortex, and the response of this hormonal system to a variety of stimuli are better understood and can be better studied as a result of his investigations.

Doctor Sayers was born in 1914. He received the degree of M.S. in physics from the University of Michigan in 1936 and the Ph.D. degree in physiological chemistry from Yale University in 1943. From 1943 to 1945 he served with the Office of Scientific Research and Development at Yale University and in 1945 became Assistant Professor of Pharmacology at the University of Utah.

ERRATA NOTICE

In the paper by Hansen, entitled "A Modified Pettenkofer Reaction for the Quantitative Estimation of Dehydroisoandrosterone and Its Application to Analysis of Urinary Extracts and Fractionations" which appeared in *Endocrinology* 44: 492 (June) 1949, the following error has been noted:

On page 492, footnote 1, should read:

¹ The author is indebted to Dr. Wm. H. Stoner of the Schering Corporation, Bloomfield, N.J., and to Dr. E. Oppenheimer of the Ciba Corporation, Summit, N.J. for the steroids, to Dr. W. H. Pearlman of Jefferson Medical College for the bile acids, and to Dr. A. Rakoff of Jefferson Medical College for the clinical material employed in this investigation.

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TESTOSTERONE AND TISSUE RESPIRATION OF THE CASTRATE MALE RAT WITH A POSSIBLE TEST FOR MYOTROPHIC ACTIVITY¹

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ALTHOUGH the general metabolic effects of gonadal steroids have long been recognized, the mechanisms by which they exert these effects are as yet poorly understood. Jensen and Tenenbaum, in their 1944 review of endocrine enzyme relationships, noted that there were no reports on the relations of these hormones to specific enzyme systems *in vitro*. Since that time Kochakian has extensively studied the effects of castration and of various steroids upon the acid and alkaline phosphatases and arginases in several tissues (Kochakian, 1947). On the basis of these studies Kochakian (1946) has suggested that kidney arginase may be associated with the protein-anabolic properties of the steroids. Clark, Kochakian, and Fox (1943) have found that testosterone increases the d-amino acid oxidase of the mouse kidney. Kochakian (1943) has also showed a similar increase in the activity of this enzyme in liver. Everett and Sawyer (1946) have found that testosterone lowers the serum content of non-specific cholinesterase in the rat. McShan, Meyer, and Erway (1947) have studied the effect of various soluble steroids upon succinoxidase systems. They found no effect from the sodium sulfate conjugates of the androgens androsterone and isodehydro-androsterone. Erway, Meyer, and McShan (1947) have likewise studied malic dehydrogenase, which was markedly depressed by several synthetic estrogens, but was not affected by sodium androsterone sulfate.

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¹ This investigation was supported (in part) by a Research Grant from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service, and a grant from Ciba Pharmaceutical Products, Inc., Summit, New Jersey.

crease in sensitivity of the muscle from castrate rats as was found with the other tissues. Inasmuch as the initial QO_2 's of levator ani from all three groups of animals were the same, the identical inhibition of the respiration of this muscle by testosterone *in vitro* is probably explainable on the basis that the QO_2 of the very thin muscle from the cas-

TABLE 1. OXYGEN UPTAKE OF LIVER, DIAPHRAGM AND LEVATOR ANI OF NORMAL, CASTRATE, AND TESTOSTERONE-TREATED CASTRATE RATS

Tissue	QO_2 during first 90'	QO_2 at end of 180'	QO_2 at end of 180'—testosterone added at 90'	% of inhibition
<i>Liver</i>				
Normal				
EGR	6.5 ± 0.2 (32)*	5.3 ± 0.7 (8)	2.7 ± 0.1 (24)	49
ER	6.1 ± 0.2 (28)	4.7 ± 0.3 (8)	2.2 ± 0.1 (20)	53
Castrate				
EGR	5.2 ± 0.2 (34)	3.8 ± 0.6 (10)	3.0 ± 0.2 (24)	21
ER	5.7 ± 0.1 (28)	4.1 ± 0.2 (10)	3.0 ± 0.1 (18)	27
Castrate-treated				
EGR	4.5 ± 0.3 (33)	3.4 ± 0.5 (12)	1.9 ± 0.2 (21)	44
ER	4.8 ± 0.2 (34)	3.7 ± 0.4 (12)	1.7 ± 0.1 (23)	54
<i>Diaphragm</i>				
Normal				
EGR	6.3 ± 0.1 (30)	5.8 ± 0.5 (6)	3.2 ± 0.1 (24)	45
ER	6.3 ± 0.1 (28)	5.7 ± 0.4 (6)	3.1 ± 0.1 (22)	46
Castrate				
EGR	5.9 ± 0.1 (35)	5.6 ± 0.1 (12)	3.8 ± 0.1 (23)	32
ER	6.1 ± 0.1 (30)	5.2 ± 0.2 (8)	3.7 ± 0.1 (22)	29
Castrate-treated				
EGR	5.9 ± 0.1 (29)	5.4 ± 0.4 (8)	3.0 ± 0.1 (21)	44
ER	5.5 ± 0.1 (30)	5.0 ± 0.3 (8)	2.7 ± 0.1 (22)	46
<i>Levator Ani</i>				
Normal				
EGR	3.5 ± 0.4 (23)	2.5 ± 0.5 (5)	1.4 ± 0.1 (18)	44
Castrate				
EGR	3.4 ± 0.2 (14)	3.7 ± 0.3 (5)	2.1 ± 0.2 (9)	43
Castrate-treated				
EGR	3.2 ± 0.1 (23)	2.0 ± 0.1 (7)	1.1 ± 0.1 (16)	45

* Figures are the mean \pm standard deviation of the mean; figures in parentheses indicate the number of vessels.

trate rats was better maintained over the three hour experimental period than that of the thicker muscles from normal and castrate-treated rats (see Table 1). This question will be studied in future experiments.

The weight changes are listed in Table 2. A marked reduction in weight of the levator ani is evident in the castrate rat. Treatment with testosterone not only prevented this atrophy but produced an increase in weight beyond the normal range. Wet:dry ratios (Table 2) of liver and diaphragm were unchanged by castration but lowered in

the case of levator ani. The maintenance of protein plus protein bound water by testosterone is adequate to explain the increased wet: dry ratio of the muscle of the treated castrate. The slight discrepancy can be explained by the persistence of connective tissue elements.

Studies on brain not previously reported include the observation that testosterone inhibits pyruvate oxidation by brain cell suspensions to the same degree as it inhibits glucose oxidation. This indicates that the primary inhibitory action of testosterone is not in the fermentative phase of glucose utilization. In addition, four phenanthrene car-

TABLE 2. EFFECT OF CASTRATION AND OF TESTOSTERONE UPON BODY AND ORGAN WEIGHTS

Group	No.	Body Weight in grams	Levator ani			Diaphragm		Liver
			Wet wgt. in mg.	W:D*	% of normal wet wgt.	Wet wgt. in mg.	W:D*	W:D*
Normal	12	182 ± 8.1†	81 ± 6	4.9	100	304 ± 11	4.5	3.4
Castrate	16	161 ± 4.1	37 ± 3	4.2	46	267 ± 12	4.3	3.2
Castrate-treated	13	166 ± 8.3	147 ± 9	5.3	181	393 ± 19	4.2	3.2

* W:D is the ratio of wet weight to dry weight.

† Figures are the mean ± standard deviation of the mean.

cinogens added to brain cell suspension had no effect on oxygen uptake whatsoever.

DISCUSSION

The data presented above and in previous reports show that suspensions of testosterone inhibit the oxygen uptake of rat brain, liver and striated muscle preparations as determined by the direct method of Warburg. The effect is specific and not due to the steroidal nature of the molecule since several other phenanthrenes in the same amounts and under the same conditions do not inhibit the oxygen uptake of brain homogenates. In addition, the inhibition reported for other steroids differs quantitatively from testosterone and from compound to compound. Studies on brain preparations to elucidate the site of action indicate: 1) the cytochromes are not involved since amounts of testosterone which inhibit glucose oxidation do not interfere with the oxidation of succinate; 2) inhibition is not primarily in the fermentative phase of glucose utilization since pyruvate oxidation is inhibited as effectively as glucose oxidation, and 3) the primary site of inhibition is presumably at the dehydrogenase level of the main line of biological oxidation since inhibition of glucose oxidation is not reversed by methylene blue.

Although these findings cannot be directly transferred to the in-

tact animal because the amount of testosterone which penetrated the tissue is unknown, other evidence suggests that testosterone may be implicated in *in vivo* oxidative processes. First, brain from castrated rats has a greater rate of oxygen consumption than brain from normal rats or testosterone-treated castrates, and second, tissue from castrated animals is less sensitive to the action of testosterone suspensions *in vitro*. The hypothetical oxidative system in which testosterone is thought to play a part seems to "atrophy" after castration, such "atrophy" being prevented by administration of testosterone to the castrate animal. It is of interest that Davis, Meyer, and McShan (1949) report decreased succinic dehydrogenase and cytochrome oxidase activity in the seminal vesicle and prostate of the rat following castration and that the administration of androgen maintains these factors at the normal level. This correlates directly with the lower QO_2 of prostate incident to castration reported by Guzman Barron and Huggins.

The elevation of brain QO_2 following castration might be related to an increase in brain hexokinase activity as a result of castration such as is reported following hypophysectomy (Reiss and Rees, 1947) but this alone will not explain all the findings. Integration of the information obtained from the four tissues studied makes it seem likely that some as yet unidentified enzyme system degenerates in the absence of testosterone. In brain respiring in EGR this is manifested as the removal of a metabolic "brake" as well as by a decreased sensitivity to testosterone *in vitro*. In liver and striated muscle, which can respire equally well in ER or EGR, the decreased sensitivity to testosterone persists. The lack of rise in QO_2 of liver and striated muscle following castration, in contrast to brain, would seem to be linked to the ability of these tissues to use endogenous substrates.

The changes in weight of the levator ani confirm the work of Wainman and Shipounoff on the relation of testosterone to the striated perineal musculature. Since this muscle is more responsive to castration and testosterone than other striated muscles in the rat, it is possible that it may be used as an index of the myotrophic effect of the steroid hormones. Studies on the specificity and reliability of this effect as an indicator of general protein anabolic activity are now in progress.

SUMMARY

The oxygen uptake of liver, diaphragm, and levator ani is not increased by castration as it is in the case of brain.

Castration renders diaphragm and liver relatively insensitive to the oxidation-inhibiting property of testosterone regardless of the presence or absence of glucose. A normal degree of reactivity is preserved in the castrate rat treated with testosterone.

Castration produces marked atrophy of the levator ani muscle of the male rat. Testosterone prevents this atrophy and, in the doses

used, produces an hypertrophy. The possible use of this muscle as an indicator of myotrophic activity is discussed.

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THE MECHANISM OF ACTION OF *LITHOSPERMUM RUDERALE*

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THE ESTABLISHMENT of the activity of *Lithospermum ruderale* as a substance which produces pronounced suppression of the female reproductive cycle in mice (Cranston, 1945) has resulted in several inquiries into the exact mechanism whereby it produces its effect (Drasher and Zahl, 1946; Zahl, 1948). All of these investigations have led to the conclusion that *Lithospermum* acts by a direct suppression of the power of the anterior pituitary to produce adequate amounts of gonadotrophins. More recently, Cranston and Robinson (1949) have shown that the pituitaries of female mice which have received *Lithospermum* for a week or more show a marked reduction of gonadotrophin potency as was demonstrated by assay on immature female mice. Somewhat similar work has been in progress in this laboratory and, therefore, it seems advisable to confirm the experiments of Cranston and Robinson at this time as well as to bring forth evidence for the possible differential effect of *Lithospermum* upon the two major gonadotrophic secretions of the pituitary, the follicle-stimulating hormone (FSH) and the luteinizing hormone (LH). Such a differential effect would be of considerable significance in the proposed clinical use of this drug.

Because of the possibility of other factors (dietary or endocrine) producing inhibition of the pituitary, it was deemed necessary to investigate these avenues more completely than had been done previously before concluding that *Lithospermum* exerts its effect directly upon the pituitary.

Previous work has adequately eliminated the possibility of a deficiency of vitamins A, C, D, or E being indirectly responsible for the anterior pituitary inhibition which attends *Lithospermum* administration. Evans and Simpson (1930) have reported however that vitamin B deficiency results in a decrease of pituitary gonadotrophin potency in the rat. Despite the fact that animals on a *Lithospermum* diet exhibit none of the gross symptoms of such a deficiency, it is conceivable the

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² Thanks are hereby extended to Dr. W. R. Breneman of this department for his interest and many helpful suggestions throughout the course of this work.

pituitary might have a threshold so low that a borderline deficiency of this vitamin complex could produce inhibition of secretory activity. The members of the B complex are intimately associated with the cellular enzyme systems, and since such enzyme systems must be linked closely with the chain of events leading to the production of the secretory products of the pituitary cells, the effect of vitamin B supplementation was investigated, the results of which are reported below.

Chu (1944) has reported that the pituitaries of thyroidectomized rabbits are free of LH but contain increased amounts of FSH. The possibility of a similar differential effect of *Lithospermum* discussed below, made it advisable to rule out thyroid implication by more precise methods than had been used to date. The determination of basal metabolic rate by Cranston (1945) and histological examination by Zahl (1948) may be considered insufficiently sensitive measures of thyroid activity. Accordingly, histometric analysis of the epithelium of the thyroid follicle was undertaken.

EXPERIMENTAL

Young female mice of the Swiss strain approximately 2½ months of age were used, except for the work on vitamin B supplementation where animals 7–8 months of age were employed. A period of at least two weeks was allowed to elapse before experimental procedures were begun, during which time all animals showing extreme irregularity of estrous cycles were discarded.

The *Lithospermum* used was obtained from the Haskins Laboratories, New York City and was part of that picked in Montana during the 1948 season. The whole plant was fresh-frozen until used except for a brief period when it was removed from refrigeration during shipment to this laboratory, and during which period it underwent "fermentation" of unknown type with considerable heat production. Assay showed this material to be active when fed to mice at a level of 7% dry weight, mixed with Purina Laboratory Chow and baked into pellets under infra-red light. This level of administration was used for the work on the pituitary potency of normal and castrated animals. Later the potency of the *Lithospermum* declined and for the vitamin B supplementation work it was necessary to use a level as high as 30–40% dry weight. Animals received diet and water ad libitum.

The vitamin B supplement used³ consisted of the following mixture: thiamine HCl, 0.8 mg.; niacin, 4.0 mg.; riboflavin, 1.6 mg.; pyridoxin, 0.8 mg.; and calcium pantothenate, 4.4 mg. A total of .225 mg. of this mixture was given to each animal daily per os in aqueous solution by means of a calibrated dropper.

Castrations were performed bilaterally in one step. Vaginal smears

³ The author is indebted to Dr. H. G. Day of the Biochemistry Department, Indiana University for supplying these vitamins.

were made daily by the lavage method. Animals were sacrificed by decapitation under light Nembutal anaesthesia and the organs removed, weighed, and fixed immediately. Pituitaries were weighed moist and then acetone-dried and assayed on immature 19 day old female mice according to the method of Riley and Fraps (1942), sacrificing after a period of four days and saving uteri, vaginae, and thyroids for histological examination. Thyroids were sectioned at 5 micra, stained with hematoxylin-eosin-azure, and the height of the follicular cells measured under oil immersion with the aid of an ocular microm-

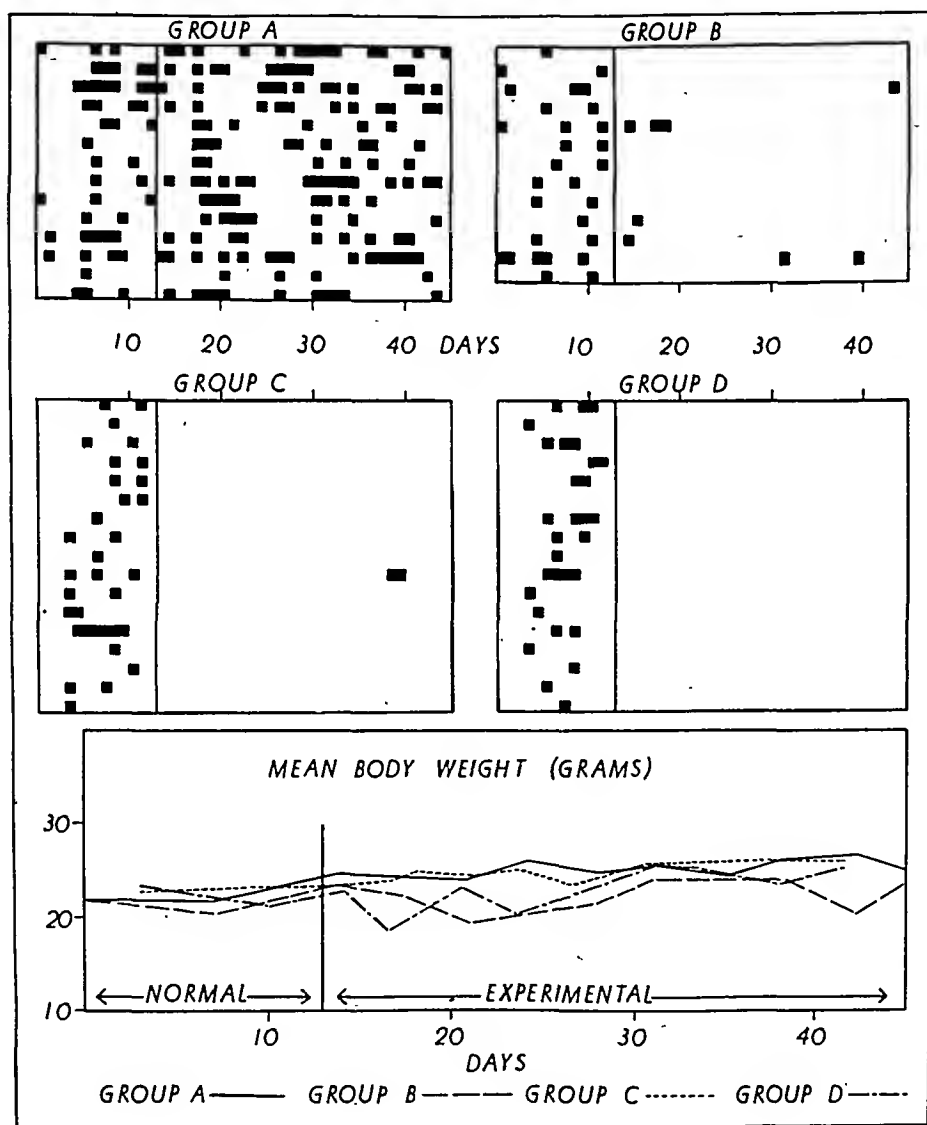


FIG. 1. Vaginal smear and body weight data from intact and castrated animals on normal and *Lithospermum* diet. Group A—intact, normal chow; Group B—intact, *Lithospermum* diet; Group C—castrated, normal chow; Group D—castrated, *Lithospermum* diet. Blackened squares indicate vaginal cornification. The period before the heavy vertical line indicates preliminary control period.

eter. Five animals were chosen at random from each of the experimental groups for this analysis, forty cells being measured in each animal.

RESULTS

Fig. 1 shows the result of *Lithospermum* administration upon the estrous cycles of normal and castrated female mice. It will be noted that this level of *Lithospermum* effectively inhibits the normal cycle, except for the recurrence of estrus in two of the animals toward the end of the experimental period (Group B) which may be due to the phenomenon of refractoriness mentioned in an earlier paper (Drasher and Zahl, 1946). The mean body weights of these animals are seen to fluctuate in an insignificant manner throughout the experiment. At the end of an experimental period of 31 days the animals were sacrificed. Organ weights of these animals are shown in Table 1 in terms of milligrams per 100 grams of body weight to correct for weight discrepancy. Weights of the ovaries and uteri of the intact animals are decreased significantly by *Lithospermum*. Adrenals, thyroids, and thymus show no change. Among the castrated animals (Groups C and D) the only organ exhibiting a significant change is the thymus which has increased in weight as would be expected following removal of estrogenic inhibition. However, it will be noted that the thymic weight of the castrated animals on *Lithospermum* diet is significantly lower than the thymic weight of the same type of animal on a normal diet. This difference is significant at only the 5% level and may be due to some other factor, such as slight inanition. Pituitary weights are given before and after acetone drying and, since the pituitaries were pooled for weighing, the figure given is a mean, uncorrected for body weight. This pooling does not lend itself well to reliable statistical analysis and for that reason no such analysis has been made. The decrease in the pituitary weights of intact animals receiving *Lithospermum* is apparent and is similar to that obtained by Cranston and Robinson (1949). However, it is of interest to note that the pituitary weights of both groups of castrated animals, while exhibiting the typical post-castration rise in weight, do so uniformly and without giving evidence of the inhibitory effect of *Lithospermum* which would be expected if this material were inhibiting gonadotrophin production.

The results of assay for gonadotrophin content of these pituitaries are shown in Table 2. The animals received the total dosage of acetone-dried powder of 0.4 mg. Later it was considered necessary to correct the weights obtained in terms of a dosage of equivalent number of pituitaries, so that assay animals receiving pituitary powder from castrated donors received the equivalent of one gland, and those receiving pituitary powder from intact donors received the equivalent of two glands. On this basis, it is obvious from the table that the results obtained by assay bear out the data on pituitary weights. Uteri from animals receiving pituitaries from normal intact animals are sig-

TABLE 1. ORGAN WEIGHTS FROM INTACT AND CASTRATED ANIMALS ON NORMAL AND *Lithospermum* DIETS

Group	Number of Animals	Ovaries	Uteri Vagina	Adrenals	Thyroids	Thymus	Pituitary	
							Wet	Dry
Control	14	46.07 (3.51)	261.50 (31.02)	53.28 (2.43)	32.00 (2.78)	221.41 (16.86)	2.8	.34
7% LS	12	** 31.00 (2.49)	** 146.91 (13.79)	55.58 (1.02)	26.41 (1.94)	194.55 (15.11)	1.9	.20
Castrated Controls	17	—	82.17 (5.49)	51.29 (2.39)	29.00 (1.80)	** 309.58 (14.56)	3.9	.40
Castrated 7% LS	17	—	95.70 (4.83)	61.35 (2.27)	30.70 (1.78)	* 269.06 (10.66)	3.5	.38

Weights in this table are in mg. per 100 g. body weight, except pituitary weights which are unadjusted. ** indicates a significant difference beyond the 1% level, * a difference at the 5% level as calculated by the "z-test" for significance.⁴ Figures in parentheses following the weights indicate the standard error of the mean.

⁴ R. A. Fisher's transformation to test significance of differences of standard deviations when N's are small. See Croxton, F. E. and Cowden, D. J., *Applied General Statistics* pp. 344-359, Prentice Hall, New York, 1945.

nificantly heavier than those from assay animals receiving pituitaries from intact *Lithospermum*-fed animals. It is again apparent that *Lithospermum* administration produces no effect on the elevated gonadotrophin potency of the pituitary of the castrated animal, since the uteri from assay animals injected with the pituitaries from castrated animals on either experimental or normal diets show an increase in weight of similar magnitude. Because of this superficial paradox, the ovaries and uteri of both donor and assay animals were studied carefully.

Histological examination of the ovaries of the intact donor animals which had received *Lithospermum* showed smaller ovaries than those

TABLE 2. ORGAN WEIGHTS FROM ASSAY ANIMALS RECEIVING PITUITARIES FROM INTACT AND CASTRATED MICE ON NORMAL AND *Lithospermum* DIETS

Group	Number of animals	Ovaries	Uterus vagina	Thyroids
Blank	6	6.23 (0.54)	17.81 (1.58)	5.91 (0.42)
Control	6	**8.32 (0.37)	**23.30 (1.75)	5.68 (0.35)
7% LS	6	5.78 (0.54)	20.06 (1.00)	5.00 (0.54)
Blank	7	**5.81 (0.50)	**15.81 (0.69)	4.93 (0.12)
Castrated Control	8	7.53 (0.57)	63.02 (3.61)	4.06 (0.33)
Castrated 7% LS	7	8.25 (1.03)	60.02 (2.27)	4.21 (0.57)

Weights in this table are in mgs., unadjusted for body weight. ** Indicates statistical significance beyond the 1% level by the "z-test." Figures in parentheses following the weights indicate the standard error of the mean.

from animals on a normal diet. However, the ovaries from *Lithospermum*-fed animals contained what appeared to be completely functional follicles with no sign of the atresia reported earlier (Drasher and Zahl, 1946). The only difference between the ovaries of these two groups was the complete absence of luteinization in the ovaries of the *Lithospermum*-fed animals. Examination of the uteri gave results which correlated well with the picture obtained in the ovaries. Uteri were graded with respect to degree of glandular development on a scale of one to four, grades 3 and 4 showing definite progestational development. The uteri of the *Lithospermum*-fed animals, of course, had some uteri classified as either grade 1 or 2 (actually only two of the animals) but in these cases examination of both the estrous smear and ovarian histology indicated the estrogenic phase of the estrous cycle.

The ovaries of the assay animals which received pituitary powder from both *Lithospermum*-fed and normal diet castrated animals showed follicular development beyond that found in the assay blanks, but had not undergone luteinization. Likewise, the uteri of these animals did not give evidence of extreme glandular development, although thickness of the endometrium was increased. The vaginae of the assay animals injected with pituitary powder from intact animals, normal diet or *Lithospermum*-fed, showed early stages of stimulation

with no cornification of the epithelium, while vaginae from animals injected with pituitary powder from both groups of castrated animals exhibited complete epithelial cornification.

The results of the histometric analysis of the donor thyroids are presented in Table 3. The results of this analysis indicate that thyroid

TABLE 3. HISTOMETRIC ANALYSIS OF THE FOLLICULAR EPITHELIUM OF THYROIDS FROM INTACT AND CASTRATED ANIMALS RECEIVING NORMAL AND *Lithospermum* DIETS

$$F = \frac{66.65}{45.47} = 1.465 \text{ (not significant)}$$

	Degrees of freedom	Sum of squares	Mean square
Variance between cells	780	3547.0	45.47
Variance between animals	16	789.47	49.34
Variance between treatments	3	199.97	66.65

The method employed in this analysis was that of Snedecor (1948) for the analysis of variance with multiple classification.

implication can be conclusively eliminated from consideration since no significant difference in the heights of the follicular cells could be detected.

The results of vitamin B supplementation are shown in Fig. 2. Group C, receiving *Lithospermum* alone, shows a definite inhibition of estrous cycles when compared with the two control groups, A and B, receiving normal chow and normal chow plus vitamin B supplement respectively. This inhibition is not as complete as that obtained in the earlier series (Fig. 1) and is probably due to the decrease in *Lithospermum* activity mentioned earlier. That excess (adequate B complex was supplied in the normal chow of which the experimental diet was largely composed) vitamin B does not decrease the effectiveness of *Lithospermum* administration, is evident.

DISCUSSION

Results presented above indicate that some explanation of *Lithospermum* activity other than a complete suppression of gonadotrophin production must be found. Indeed, the fact that treated animals return to normal estrous cycles with ease when removed from such a diet even after having been fed *Lithospermum* for relatively long periods of time (Cranston and Robinson, 1949) would cast doubt upon such a hypothesis. The presence of normal follicles with well-developed antra in *Lithospermum*-fed animals suggests that the pituitary is not completely functionless. That *Lithospermum* does not inhibit the post-castration rise in gonadotrophins is further evidence of this. The presence of a complete diestrus in treated animals argues for the lack of estrogenic stimulation, and on the basis of generally accepted ideas of pituitary control of ovarian secretion, viz. that FSH induces secretion of estrogen, LH induces secretion of progesterone, this would point to a suppression of the production of both FSH and LH.

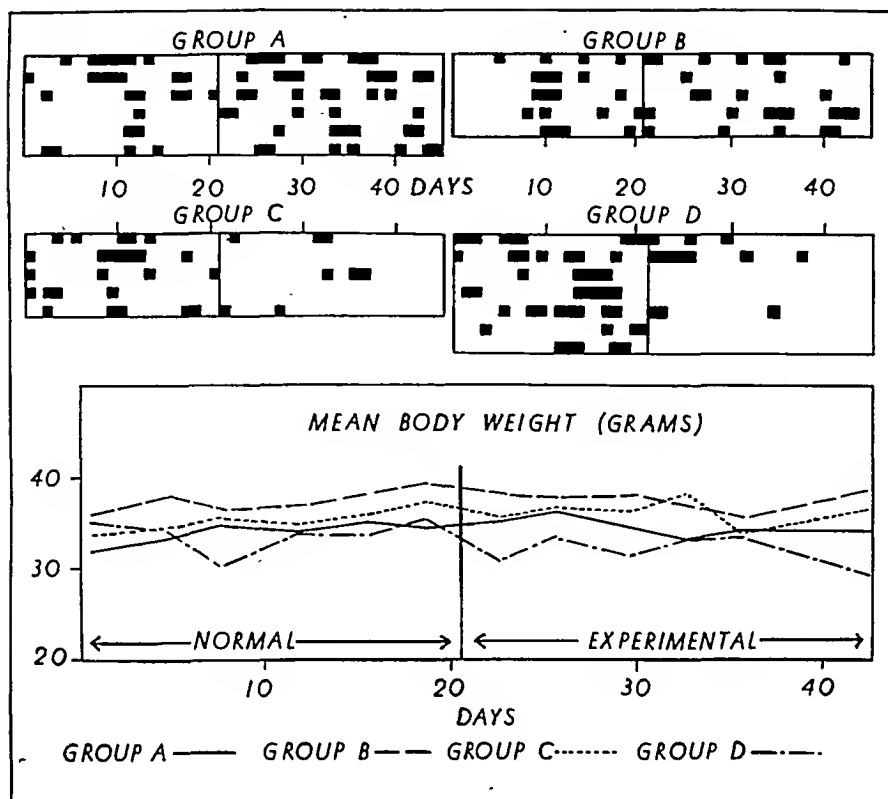


FIG. 2. Vaginal smear and body weight data from intact animals showing the effect of vitamin B supplementation on *Lithospermum* administration. Group A—normal chow; Group B—normal chow, vitamin B supplement; Group C—*Lithospermum* diet; Group D—*Lithospermum* diet, vitamin B supplement. Blackened squares indicate vaginal cornification. The period before the heavy vertical line indicates preliminary control period.

However, it is widely recognized that at present our knowledge of precisely when FSH enters the picture of follicular development and to what degree synergistic action with LH is necessary for the production of significant amounts of estrogen, is incomplete. This is largely due to the difficulty which the proteinaceous nature of the pituitary hormones presents to adequate and accurate fractionation into FSH and LH components. Greep, van Dyke, and Chow (1942) using highly purified thylakentrin (FSH) have shown that in hypophysectomized rats FSH stimulation alone does not produce secretion of estrogen by the ovary. However, the presence of ovarian follicles containing definite antra is evidence of FSH stimulation. This is precisely the picture encountered in the animals reported upon above.

Hellbaum and Greep (1940) have studied the changes in the pituitaries of rats following castration in a quantitative manner and found that although an increase in gonadotrophin potency occurs shortly thereafter, this increase is largely due to an accumulation of FSH, LH

not being present in detectable amounts until 15 days after castration at the earliest. Since the animals reported upon above were castrated for only 31 days, it is likely that LH would not be expected to be present in large amounts and any decrease in the LH content of the pituitaries of the castrated, *Lithospermum*-fed animals would not be easily demonstrable. Histological examination of the ovaries of assay animals receiving such pituitaries also gives evidence for a predominantly FSH stimulation, no luteinization being found.

A consideration of the above has led to the hypothesis that *Lithospermum* exerts its effect by a specific inhibitory action upon the production of the luteinizing hormone of the anterior pituitary, allowing the secretion of the follicle-stimulating hormone to remain close to normal. This would account for the maintenance of the ovary in a pre-ovulatory state so that ovulation and normal estrous cycles could be resumed rapidly after the cessation of *Lithospermum* treatment, as is the case. Likewise, if it is assumed that small amounts of LH must be present to act synergistically with FSH for the secretion of estrogens by the ovary in amounts sufficient to produce vaginal cornification, the persistent state of diestrus in *Lithospermum*-fed animals can be explained without necessitating the postulation of a reduced FSH output by the pituitary. Whether this hypothesis is correct can only be shown by further experimentation on the quantitative changes in the gonadotrophin potency of animals castrated for periods longer than one month. Such work is now in progress.

SUMMARY

Lithospermum ruderdale was fed to normal and castrated animals. While it was found to decrease the gonadotrophin titer of the pituitaries of intact animals, no such diminution was observed in the characteristically elevated gonadotrophin potency of the pituitaries of the castrated animals.

The possibility of indirect action via the thyroids or a borderline deficiency of the vitamin B complex has been negated.

The hypothesis of *Lithospermum* action by means of a specific inhibition of the production of the luteinizing hormone of the anterior pituitary has been suggested.

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THE EFFECT OF GROWTH HORMONE PREPARATIONS ON ALKALINE PHOSPHATASE OF THE TIBIA

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EVIDENCE that anterior pituitary growth hormone stimulates growth of bone is quite extensive. For example, Young (1945) obtained a 6% increase in tibial length by injecting a growth preparation for 60 days into plateaued female rats fed *ad lib.* Becks, *et al.* (1946) found that administration of growth hormone to hypophysectomized rats stimulated bone formation, even after a postoperative interval of one year. The width of the proximal epiphyseal cartilage of the tibia of hypophysectomized rats is used to assay the growth hormone (Evans, *et al.* 1943). Related evidence is the fall in urinary phosphate in dogs (Gaebler and Price, 1937), increase in serum inorganic phosphate in rats (Li, Geschwind, and Evans, 1949), and increase in plasma alkaline phosphatase in rats (Li, Kalman, and Evans, 1947), after growth hormone treatment. Since Robison (1932) established a relationship between bone alkaline phosphatase and bone calcification, and Gomori (1943) confirmed this relationship histochemically, it appeared of interest to investigate the effect of growth hormone upon bone alkaline phosphatase in rats.

EXPERIMENTAL

Normal and hypophysectomized¹ Sprague-Dawley rats of each sex and several age levels were used. In the preliminary experiment, and that reported in Table 1, the rats were fed Rockland rat diet. Work presented in Tables 2 and 3 was carried out with the diet of Bennett, *et al.* (1948). During the final experiment (Table 4), the diet consisted of: casein 24%, white flour 55.5%, whole milk powder 10%, CaCO₃ 1.5%, NaCl 1%, and corn oil 8%. Vitamins were added according to Gordan, *et al.* (1948), except that vitamins A and D were given as 2 ml. of haliver oil (60,000 U.S.P. vitamin A units and about 1000 U.S.P. vitamin D units per gm.) per kg. of diet. Both *ad lib.* and paired-feeding techniques were employed, as indicated in the text.

Growth hormone preparations² were obtained as lyophilized

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¹ The hypophysectomized rats were obtained from the Hormone Assay Laboratory, Inc., Chicago, Illinois.

² The authors are indebted to Dr. D. A. McGinty and Mr. L. W. Donaldson of Parke, Davis & Company for three lots of growth preparation, which had been assayed on plateaued rats. These preparations are about one half as active as the most potent ones obtained by the above investigators to date.

TABLE 1. THE EFFECT OF LARGE DOSES OF GROWTH HORMONE ON TIBIA WEIGHT AND ALKALINE PHOSPHATASE OF MATURE MALE RATS

	No. of rats	Average wet wt. of tibia in gm.	Average alkaline phosphatase concentration of tibia in U./gm.
Injected*	3	0.806	17.6
Control	5	0.804	21.2

* 10 mg. growth preparation (1740 R.U./gm., proven quite potent in promoting nitrogen storage in dogs), was injected daily per rat for 14 days.

powders. For use, this material was suspended in 0.8% saline and injected intraperitoneally. Solutions were prepared fresh every third day and kept frozen at -18°C . when not in use.

All rats were sacrificed at the end of an experiment by a blow on the head, followed by decapitation and exsanguination. The hind legs were amputated and stored at -18°C ., the muscle being left in place to prevent dehydration. For analysis, the tibias were dissected out, weighed immediately (wet weight), and reduced to small fragments, either by grinding in a mortar with 1 gm. of quartz sand and 1 to 2 ml. of water, or by freezing with liquid air in a steel mortar and pulverizing with a sharp blow on the pestle. This material was transferred to a graduated tube with the aid of distilled water and diluted, the final volume in ml. being 10 times the number of tibias pooled. Four drops of chloroform were added for each 10 ml. of final solution. The tube was tightly stoppered, and the contents allowed to autolyze at room temperature for 1 to 2 weeks with daily inversion of the

TABLE 2. THE EFFECT OF GROWTH HORMONE ON TIBIA WEIGHT AND ALKALINE PHOSPHATASE OF IMMATURE HYPOPHYSECTOMIZED* FEMALE RATS

	No. of rats	Average initial body wt. in gm.	Average wt. gain in gm.	Average tibia wt. in gm.	Average tibia alkaline phosphatase in U./gm.
I. Hypophysectomized†					
A. Treated	6	154	52.8	0.402	31.6
B. Control	5	146	- 1.2	0.337	18.7
II. Normal					
A. Treated	5	175	24.6	0.419	27.3
B. Control	5	165	- 0.6	0.405	23.9
P (IA, IB)†			<0.01	<0.01	
(IIA, IIB)			<0.01	>0.3	

* Two months old at operation. Treatment with 1 mg. growth hormone daily per rat (1384 R.U./gm.) for 17 days started 2 weeks after the operation.

† Group IA and IB were fed *ad lib*. Group IIA was pair-fed against Group IA, and Group IIB was pair-fed against Group IB.

‡ Fisher's (1948) table of t. The symbols in parenthesis indicate the groups compared.

|| Weight at start of injection period.

TABLE 3. THE EFFECT OF GROWTH HORMONE ON TIBIA WEIGHT AND ALKALINE PHOSPHATASE OF IMMATURE, PAIR-FED, HYPOPHYSECTOMIZED* FEMALE RATS

	No. of rats	Average initial body wt.†	Average wt. gain in gm.	Average tibia wet wt. in gm.	Average tibia alkaline phosphatase in U./gm.
I. Hypophysectomized					
A. Treated	5	176	50.0	0.437	32.4
B. Control	7	173	4.0	0.389	15.5
II. Normal					
A. Treated	5	189	25.0	0.459	24.4
B. Control	6	191	5.0	0.464	25.6
P (IA, IB)†				<0.01	
(IIA, IIB)				>0.7	

* Treatment same as in Table 2, except that a 24 day injection period was employed and all groups were pair-fed against group IB.

† Same significance as in Table 2.

‡ Weight at start of injection period.

tubes. Samples were taken several times during this period to obtain results at maximum extraction. After suitable dilution of this stock enzyme solution, phosphatase activity was determined by the King-Armstrong procedure as modified by Kochakian (1945), the pH of the substrate being 9.1. A Coleman Junior photoelectric colorimeter was employed to read the optical densities at a wave-length of 660 mμ. Activity units are expressed in terms of mg. phenol liberated/hr.

RESULTS

In a preliminary experiment on plateaued female rats, 3 injected animals were restricted to the food intake of 3 untreated controls. Each of the former received 6 mg. of growth preparation (1740 R. U./gm.) daily for 7 days. There was no significant increase in the weight or alkaline phosphatase of the tibias. In the next experiment (Table 1) 8 male rats, 8 to 10 months old and fed *ad lib.*, were used. Differences between treated and untreated animals are not informa-

TABLE 4. THE EFFECT OF SMALL DOSES OF GROWTH HORMONE ON TIBIA WEIGHT AND PHOSPHATASE OF IMMATURE, HYPOPHYSECTOMIZED,* PAIR-FED FEMALE RATS

	No. of rats	Average tibia wet weight in gm.	Average tibia alkaline phosphatase in U./gm.
Treated†	10	0.231	31.7
Control	10	0.190	23.8
P		<0.01	

* One month old at hypophysectomy. Plateaued 10 days before starting growth hormone therapy.

† 0.05 mg. growth hormone (1361 R.U./gm.)/rat/day for 6 days, then 0.1 mg./rat/day for 5 days.

tive, as regards tibial weight and alkaline phosphatase. The increase in body weight is not decisive. This is in agreement with the well-known refractoriness of the male rat to growth hormone treatment (Evans and Simpson, 1931). In our limited series, plateaued animals of either sex did not respond quickly with respect to tibial weight. It was, therefore, decided to carry out the rest of the investigation on the much more sensitive, immature hypophysectomized animal.

Results of an experiment, using normal and hypophysectomized female rats, two months old, are recorded in Table 2. The increase in wet weight and alkaline phosphatase of the tibia in the hypophysectomized animals treated with growth hormone is quite striking. Alkaline phosphatase almost doubled, while the tibial weight increase was equal to 27 days of normal growth in the controls. Normals which were pair-fed against the two groups of hypophysectomized animals show much smaller differences, which, as will be shown later, might well be due to the difference in food intake. Group IIA received on an average 2 grams more food per day during the injection period than the untreated normal group IIB. It is to be noted that the hypophysectomized untreated animal has a bone phosphatase level lower than that of the mature males employed in the experiment reported in Table 1. This might be taken to indicate that the bone phosphatase level in these animals is below normal maintenance levels as regards bone formation. The somewhat higher value observed in the normal controls would be indicative of normal bone development in the young and growing animal.

The next experiment was designed to eliminate the variations in food intake. The four groups were arranged as in the previous experiment. Hypophysectomized controls were fed *ad lib.*, while the remaining three groups were pair-fed against this group. The results (Table 3) show the same general trend as in the previous experiment, in which the treated hypophysectomized animals were fed *ad lib.* Untreated hypophysectomized animals have the same low level of alkaline phosphatase activity. Treatment of the hypophysectomized animals doubled the concentration of phosphatase, increasing it above the normal control level. Restricted food intake had no tendency to depress the response. In keeping with the phosphatase response, the low tibia weight due to hypophysectomy was restored to a great extent during the period of growth hormone therapy. In this experiment (Table 3), there is no great difference in phosphatase concentration between the two groups of normal animals. With the food intake equal, the difference previously obtained (Table 2) was reduced and inverted.

As the dosage of growth hormone used in the above experiments was intended to give maximal responses, it might be argued that a "physiological" level of hormone therapy would show no such change. Consequently, an experiment was carried out in which each of 10 hy-

pophysectomized rats received a total of 0.8 mg. of growth hormone preparation in divided doses over a period of 11 days. From the results of this experiment (Table 4), it is obvious that the effect of growth hormone therapy on the wet weight and alkaline phosphatase concentration of the tibia occurs at the low dosage employed, which produced submaximal weight responses averaging 1.3 gm. per rat per day. This is in accord with the extreme sensitivity obtained by Kibrick, *et al.* (1941) in the assay of the growth hormone by the epiphyseal disc method.

DISCUSSION

Studies on the effects of growth hormone preparations on eight enzyme systems are in various stages of completion in this laboratory, and indicate that results of the following types may be expected: first, changes in concentration of enzymes or substrates which are readily correlated with known effects of the hormone; second, changes in which the enzyme may be regarded as an individual protein participating in a general increase in protein synthesis; third, changes having no apparent significance at the moment. Results of the present experiments are clearly in the first category. If one accepts the view of Robison (1932) regarding the role of alkaline phosphatase in bone formation, the large increase of this enzyme in the tibia which we observed in hypophysectomized rats treated with growth hormone would be anticipated, since the action of the hormone on skeletal growth is well known. The expectations of Li and Evans (1947) that the phosphatase concentration in tissues of normal and hypophysectomized rats would be increased by growth hormone are borne out in the case of bone phosphatase in hypophysectomized, but not in normal animals. Results for acid and alkaline phosphatase in liver and kidney, which will be published later, indicate that the changes in concentration of these enzymes in different tissues are not necessarily parallel.

SUMMARY

In immature hypophysectomized female rats, the alkaline phosphatase concentration in the tibia is lower than in normal mature male rats; it is greatly increased by maximal and submaximal doses of growth hormone, and may even be elevated above the level for normal growing animals of the same age and sex. The rise in tibia phosphatase concentration appears to be related to the increase in tibia weight resulting from treatment. Mature and immature normal animals do not respond significantly in these respects, under the conditions employed.

ACKNOWLEDGMENTS

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mine-glutaminase system (Bartlett, 1949) which were made available for the experiment shown in Table 4 of this paper.

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ADRENAL CHANGES PRODUCED IN RATS BY INFECTION WITH *B. TULARENSE* AND *B. COLI*¹

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IT IS WELL known that the adrenal glands respond to many infectious diseases by a depletion of their cholesterol content (Sayers, Sayers, Fry, White, and Long, 1944) and that this indicates, along with a falling adrenal ascorbic acid content, a state of increased adrenal activity and, if advanced enough, of adrenal exhaustion (Long, 1947). It is also known that infection with tularemia causes pathological changes in the adrenals (Lillie and Francis, 1936) and that these changes are compatible with the pathological picture of adrenal exhaustion (Pinchot).

Quantitative studies of the functional reserve of the adrenal cortex in tularemia, the effect of treatment with cortical hormone, and studies of the respiratory metabolism of animals infected with this disease have not been reported. It was felt that these studies might throw some light on the mechanisms by which this parasite produces its characteristic disease and symptomatology in the host. *B. coli* was used to compare the effects of tularemia with those produced by a less pathogenic gram negative micro-organism.

TECHNIQUE

Male rats of the Sprague-Dawley strain (weighing from 210–290 g.) were used throughout. They were fed a diet of Purina Dog or Laboratory Chow Checkers, supplied with water *ad libitum* and housed in a constant temperature room at $80 \pm 2^\circ \text{F}$.

In the adrenal studies the control values were obtained by removing the glands from normal animals deeply anesthetized with subcutaneously injected "Nembutal." The glands of infected animals were removed in a similar manner or very shortly after death. After removing fat and connective tissue, the glands were weighed to the nearest 0.1 mg. on a torsion balance. One gland was then used for the determination of the ascorbic acid content by the method of Roe and Kuether (1943), modified for tissues, and the other for the cholesterol determination, by the method of Schonheimer and Sperry,

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² National Research Council fellow in the Medical Sciences.

as reported by Sperry (1938). Results are reported as the average of duplicate determinations.

The *B. tularensis* organisms used were of a highly virulent strain isolated from a human case of the disease, with virulence maintained by mouse and rat passage.³ Subcultures were made on dextrose cysteine blood agar 24 hours before the animals were to be inoculated. After 24 hours growth at 37° C., the organisms were suspended in sterile physiological saline and diluted to the approximate concentration desired by turbidometric counts with an Evelyn colorimeter. Plate counts were then done on the suspensions to be injected by the method of Downs, Coriell, Chapman, and Klauber (1946).

The *B. coli* was a highly hemolytic strain isolated from the stool of a patient at the New Haven Hospital. This organism was prepared for injection by growing for 12 hours at 37° C. on beef infusion broth. The bacteria were then centrifuged out of the culture and resuspended in 5 ml. of sterile physiological saline per 100 ml. of culture. This material was injected intravenously.

In the first treatment experiment, "Wilson's Cortical Extract," each ml. of which is equivalent to 75 g. of fresh gland, was given subcutaneously in 2 ml. doses to infected rats 5 times a day. Treatment was started 44 hours after the animals were infected and continued until 96 hours after infection. All animals were fasted 96 hours starting at the time of challenge.

In the second treatment experiment Upjohn's "Lipoadrenal Extract," each ml. of which is equivalent to 2 mg. of compound E(11 dehydro 17 hydroxycorticosterone) and contains 40 rat survival-growth units, was given subcutaneously to infected rats in 0.5 ml. doses every six hours starting at 18 hours after infection and continuing until all animals were dead. Control animals of approximately equal weight (± 6 g.) received equal doses of alcohol extracted sesame oil.

The respiratory gas exchange was determined in a constant temperature room at $80 \pm 2^\circ$ F. by the method of Haldane (1892) on rats fasted for 24 hours. The animals were then allowed food for 24 hours, then infected and food was again withheld until the end of the experiment. Periodic measurements of respiratory exchange were made throughout the infection. Control animals were subjected to the same feeding and fasting regime, so as to be able to distinguish between changes produced by the infection and those produced by prolonged fasting, habituation to the procedure, and so on.

Results are reported as means and standard errors throughout.

RESULTS

Table 1 shows the effects of tularemia on the adrenals of the rat. The control values represent determinations on 14 rats except in the case of the cholesterol values which are from 11 rats only.

It will be seen that there was a marked increase in the size of the adrenals of all infected animals. There was an extreme and consistent fall in the adrenal cholesterol values, while the ascorbic acid contents varied from normal to markedly depleted values. This variation and

³ This strain, SM24R4, was made available by the Technical Director, Camp Detrick.

TABLE 1. THE EFFECTS OF VARIOUS DOSES OF *B. TULARENSE* ON THE ADRENALS OF THE RAT

Wt. g.	Number of organisms injected	Time of death or sacrifice	Mg. adrenal/100 g. body wt.	Adrenal ascorbic mgs./100 g.	Adrenal cholesterol g./100 g.	Minimum body temperature °F.
<i>14 controls</i>						
273 ± 13.1	None	—	12.6 ± 0.71	436 ± 13.4	4.71 ± 0.23	—
<i>Infected Animals</i>						
212	16,500	D 4 days	22.2	348	—	—
236	16,500	D 4 days	35.8	166	—	—
232	16,500	S 8 days	—	427	—	100.8
240	16,500	S 8 days	25.9	242	0.35	100.2
230	16,500	S 8 days	18.5	465	0.74	100.6
238	16,500	S 8 days	—	212	—	100.0
242	150,000	D 70 hours	23.3	414	0.5	—
240	150,000	D 68 hours	21.9	421	0.5	—
*218	185,000	D 74 hours	37.2	143	0.39	—
*264	185,000	D 98 hours	21.7	392	0.52	—
*230	185,000	D 98 hours	23.0	319	0.69	—
*220	1,850,000	D 98 hours	23.0	199	0.47	—
*280	1,850,000	D 74 hours	25.1	294	0.40	—
*270	1,850,000	D 91 hours	23.9	290	0.39	—
†240	1,550,000	D 48 hours	29.1	226	0.55	93.2
†256	1,550,000	D 49 hours	20.8	339	0.57	86.0
†250	1,550,000	D 66 hours	—	—	0.42	91.4
†240	1,550,000	D 66 hours	33.2	160	0.55	93.4
241			25.6 ± 1.5	297 ± 25.2	0.50 ± 0.03	

* Animals fed and kept at room temperature.

† Animals fed and kept at room temperature. These animals received sesame oil and are controls for those treated with lipoadrenal extract.

the much smaller variations in the adrenal to body weight ratio and in the cholesterol content seem to have no relation to differences in the size of the infecting dose of organisms or to survival time.

Table 2 shows the effects of treatment of infected animals with water and lipid extracts of the adrenal cortex. The aqueous extract neither prolonged the survival time of the recipients over the controls nor prevented the depletion of the adrenal cholesterol. The lipoad-

TABLE 2. THE EFFECTS OF TREATMENT OF EXPERIMENTAL TULAREMIA IN RATS WITH CORTICAL HORMONE

Wt. g.	Number of organisms injected	Time of death	Mg. adrenal per 100 g. body wt.	Adrenal ascorbic mgs./100 g.	Adrenal cholesterol g./100 g.	Cortical hormone
247	150,000	94 hours	19.7	333	0.55	aqueous
234	150,000	139.5 hours	16.8	—	0.85	extract
272	150,000	79.5 hours	14.1	289	0.66	extract
*242	1,550,000	96 hours	28.9	176	0.45	lipid
*250	1,550,000	66 hours	25.1	262	0.89	extract
*246	1,550,000	51 hours	21.6	354	0.82	extract
*242	1,550,000	66 hours	27.7	324	0.59	extract
248			22.0 ± 2.1	290 ± 26.4	0.69 ± 0.06	

* Not fasted or kept in constant temperature room.

renal extract similarly failed to prevent depletion of the glands. The survival time of the treated animals was prolonged from the control value of 57.3 ± 5.0 hours to 69.8 ± 9.5 hours in the animals receiving lipid extract, but this is not a statistically significant fact. The rats

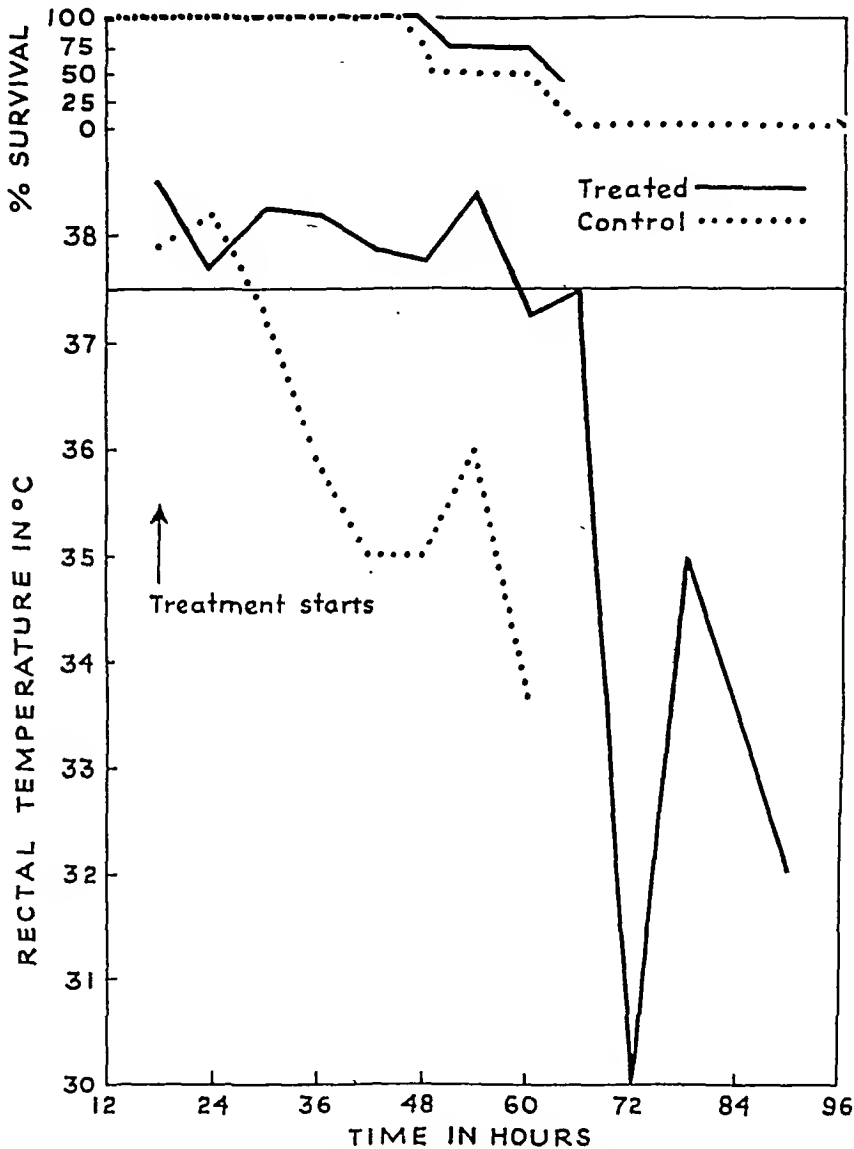


CHART 1. The results of treatment with 0.5 ml. of lipid adrenal extract 4 times per day of rats infected with *B. tularensis*.

receiving the lipoadrenal extract were, however, able to maintain their body temperature longer than those which did not receive it, as is shown in chart 1. Rats dying of tularemia have a characteristic and profound fall in body temperature, the onset of which precedes death by from 12 to 24 hours. Moreover, the rats treated with cortical hormone did not seem acutely ill until a few hours before death, while the

controls gave evidence of a severe illness 30 hours after inoculation with *B. tularensis* organisms.

There were no consistent differences in oxygen consumption or caloric output between the infected and control groups. The infected group did have consistently higher RQ values than the controls, however. Six animals were used in the control group and ten in the infected. The infected group received from 195 million to 5 billion *B. tularensis* organisms. The mean RQ's were as follows: Control determinations; controls $.719 \pm .0053$, animals to be infected $.722 \pm .0047$; 24-hour determination; controls $.732 \pm .0053$, infected $.770 \pm .0086$. *P* is less than .01; 48-hour determination; controls $.735 \pm .0054$, infected

TABLE 3. THE EFFECTS OF LIVING *B. COLI* INJECTED INTRAVENOUSLY INTO RATS

Wt. g.	Ml. of <i>B. coli</i>	Mg. adr./ 100 g.	Time sacrificed or death	Adrenal ascorbic acid mg./100 g.	Adrenal cholesterol g./100 g.	Minimum body tempera- ture °F.
220	0.5 ml.	18.1	S 5 hours	274	1.92	92.0
210	0.5 ml.	16.1	S 4.5 hours	262	2.59	91.0
210	1.0 ml.	15.3	D 5 mins.	329	3.39	—
225	1.0 ml.	17.8	S 5 hours	260	1.84	91.0
216	2.0 ml.	17.6	D 15 mins.	404	3.24	96.0
220	2.0 ml.	14.0	S 2.5 hours	265	1.28	83.5
280	2.0 ml.	12.6	S 4.5 hours	266	2.19	91.0
310	2.0 ml.	12.0	S 4.5 hours	255	2.31	88.5
316	2.0 ml.	11.5	S 3.8 hours	234	1.08	90.0
290	2.0 ml.	12.4	S 4 hours	250	1.86	90.0
259*		14.3 ± 0.94		258 ± 4.3	1.88 ± 0.18	

* The animals dying in 5 + 15 minutes after injection are excluded from the means, as the time was inadequate for their adrenals to show changes in cholesterol and ascorbic acid content.

$.783 \pm .0159$. *P* is less than .01; 72-hour determination; controls $.742 \pm .0029$, infected $.756$ (one animal surviving).

The results of inoculation with *B. coli* are shown in Table 3. The animals were sacrificed when in extremis, or when their temperatures began to rise from a low point. This was felt to represent the time of maximal change, since several were allowed to recover after their temperatures began to rise, which they did uneventfully. The inoculations then either produced death in a relatively short time or were self-limiting. It will be seen from the table that the injection of this organism produced a moderate increase in adrenal size, a moderate fall in adrenal cholesterol, and a moderate and consistent fall in adrenal ascorbic acid. There was also a fall in temperature.

DISCUSSION

The adrenal hypertrophy and the profound fall in cholesterol content in rats infected with tularemia probably indicates a marked exhaustion of the glands by this disease. The drop in cholesterol is greater than that produced by a single injection of adrenotropichor-

mone (Sayers, Sayers, Liang, and Long, 1946) and is of the same magnitude as the type III adrenal response described by Sayers, Sayers, Fry, White and Long, (1944) which represents a situation characterized by continuous stimulation and collapse ending in death. The cholesterol values are similar to those reported in humans dying of infectious diseases.

On the other hand, the mean value of the adrenal ascorbic acid content of the infected animals is higher than that observed by the above authors following a single dose of adrenotropic hormone. Some of the levels of the ascorbic acid are perfectly normal in spite of the extremely low cholesterol values and the increased adrenal size. It would therefore seem likely that the adrenal ascorbic acid content may return to normal or near normal values in some cases of prolonged adrenal stimulation. In any case it would appear that this determination alone is not a reliable index of the functional state of the adrenal which has been subjected to a prolonged stress.

It has been shown that pretreatment with cortical hormone prevents the fall in adrenal ascorbic acid and cholesterol content produced by many forms of stress, and it has been suggested that this prevents stimulation of the pituitary by the low blood level of cortical hormones otherwise presumably caused by stress (Sayers, and Sayers 1947). It has also been shown that the rat is able to restore both cholesterol and ascorbic acid levels in the adrenal to normal within 24 hours after a fall produced by adrenotropic hormone (Sayers, Sayers, Liang and Long, 1946).

Vogt (1943) has shown that the adrenals of a ten kilo dog may discharge the daily equivalent of 230 ml. of commercial water extract of the same strength as that used in these experiments. This output is presumably markedly increased by stress, yet in infectious diseases the supply is apparently not adequate to meet the demand as is shown by the exhausted glands found at death. As can be seen from the results, treatment with the available preparations of adrenal cortical hormones, at least in the amounts used in these experiments, not only failed to protect the animals, but also failed to prevent the depletion of the adrenal cholesterol. The answer to the question of whether or not larger doses would protect the adrenals and save the animals must await the availability of more potent preparations of cortical hormone, since the two used here were given in larger doses than could be completely absorbed. The temperature curve and appearance of the animals treated with the lipid preparation suggests that the treatment did help the animals in the early stages of the disease.

In any event these experiments indicate that the requirement for cortical hormone is greatly increased in tularemia. Our animals received 10 ml. of water soluble extract per day (or 400 ml. per kilo of animal for comparison with Vogt's figures) and in the other experiment the equivalent of 4 mg. of compound E or 80 rat survival-

growth units, in addition to the output of their own adrenals, without meeting the demand.

These results suggest the possibility that at least some of the published reports of failures of cortical preparations to benefit various diseases upon which they have been tried may have been the result of inadequate dosage.

The experiments with *B. coli* show the marked differences in the effects produced by a stress of short duration from those produced by a long-term stress, even when both are caused by the injection of bacteria. In the animals receiving the colon organism the levels of adrenal ascorbic acid and cholesterol were quite analogous to those seen following a single injection of pituitary adrenotropic hormone (Sayers, Sayers, Liang, and Long, 1946) or of epinephrine (Long and Fry, 1945). The adrenals showed less evidence of exhaustion as measured by hypertrophy and cholesterol changes than in the animals which received *B. tularensis* organisms. This is presumably because of the shorter duration of the adrenal stress in the former experiment, not because of a submaximal stimulus, since adrenotropic hormone does not produce any greater changes in the same time.

The uniform fall in ascorbic acid content (except in the animals dying in 5 and 15 minutes) and the close correlation between ascorbic acid and cholesterol contents show the reliability of the former determination in short experiments such as this. This is in marked contrast with the situation in the animals with tularemia. The fall in body temperature is similar to that seen in animals dying of tularemia.

SUMMARY

Rats infected with *B. tularensis* showed an increase in adrenal size and a profound fall in adrenal cholesterol content. The change in adrenal ascorbic acid content was highly variable, indicating the unsuitability of this determination as an indication of the functional state of the adrenal cortex in prolonged infections. Infected rats showed a significant rise in RQ as compared with normals.

Dosages of adrenal cortical hormone preparations many times larger than those required for maintenance of adrenalectomized animals failed to prevent the adrenal cholesterol depletion or significantly prolong the survival time of infected animals, although the treated animals were better able to maintain body temperature and seemed more active than the controls.

Rats receiving large doses of living *B. coli* intravenously had an adrenal cortical response similar to that produced by a single injection of pituitary adrenotropic hormone as measured by adrenal size, ascorbic acid and cholesterol content. The ascorbic acid levels correlated with the other measurements of adrenal cortical function in this short-term experiment.

The differences in the effects of the two organisms on the adrenals

are believed to be primarily differences in the duration of the adrenal stimulation they produced.

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THE EFFECT OF ADRENAL CORTEX EXTRACT UPON URINARY NON-PROTEIN NITROGEN AND CHANGES IN WEIGHT IN YOUNG ADRENALECTOMIZED RATS

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THE ADRENALECTOMIZED rat is unable to grow at a normal rate (Hartman and Thorn, 1930). In the present study we have tested the suggestion (Ingle, 1944) that the slowing of growth in the adrenally insufficient animal is due entirely to secondary changes, principally the reduction in intake of food. Adrenalectomized rats were given a constant caloric intake by stomach tube and received saline to drink. The administration of adrenal cortex extract was followed by a decrease in urinary non-protein nitrogen and an increase in rate of gain in weight as compared to adrenalectomized rats which did not receive the hormone.

METHODS

Male rats of the Sprague-Dawley strain were maintained on Archer Dog Pellets until they reached a weight of approximately 300 grams. At this time they were placed in metabolism cages and adapted to the force-feeding of a medium carbohydrate diet by stomach tube each morning (8:30 to 9:15 A.M.) and afternoon (4:15 to 5:00 P.M.). The technique of force-feeding and the diet (Table 1) used were modifications of those described by Reinecke, Ball and Samuels (1939). The animals were brought to a full feeding of 26 cc. per rat per day on the 5th day. After the rats had been force-fed for 2 weeks the adrenal glands were removed by the procedure of Ingle and Griffith (1942). Asepsis was successfully maintained in these operations. All of the rats were given a solution of 1 per cent sodium chloride to drink during all phases of the experiments. The experiments were done in air-conditioned rooms in which the temperature was maintained at 74 to 78 degrees F. and the humidity at 30 to 35 per cent of saturation. Beef adrenal extract (ACE) was used which was free from alcohol and represented 40 grams of gland per cc. It was administered subcutaneously in divided injections twice daily.

Beginning with the third week after adrenalectomy, 24-hour samples of urine were collected each morning prior to feeding. The urines were preserved with thymol and added citric acid (1 g. per sample) to insure the acidity of the urines for nitrogen analysis. The determination of urinary non-protein nitrogen was by the micro-Kjeldahl procedure. In Experiment 1, the determinations were made on each 24-hour sample of urine. In Experiment 2, the urines were pooled into 72-hour samples for each rat.

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EXPERIMENTS AND RESULTS

In Experiment 1 (Figure 1), 22 adrenalectomized rats were observed during a control period of 14 days. Eleven of these rats were then given 1 cc. of ACE for 7 days, 2 cc. for 7 days, 3 cc. for 7 days, and 4 cc. for 7 days. The 11 control rats were given equal volumes of 0.9 per cent sodium chloride solution for these periods. There was a

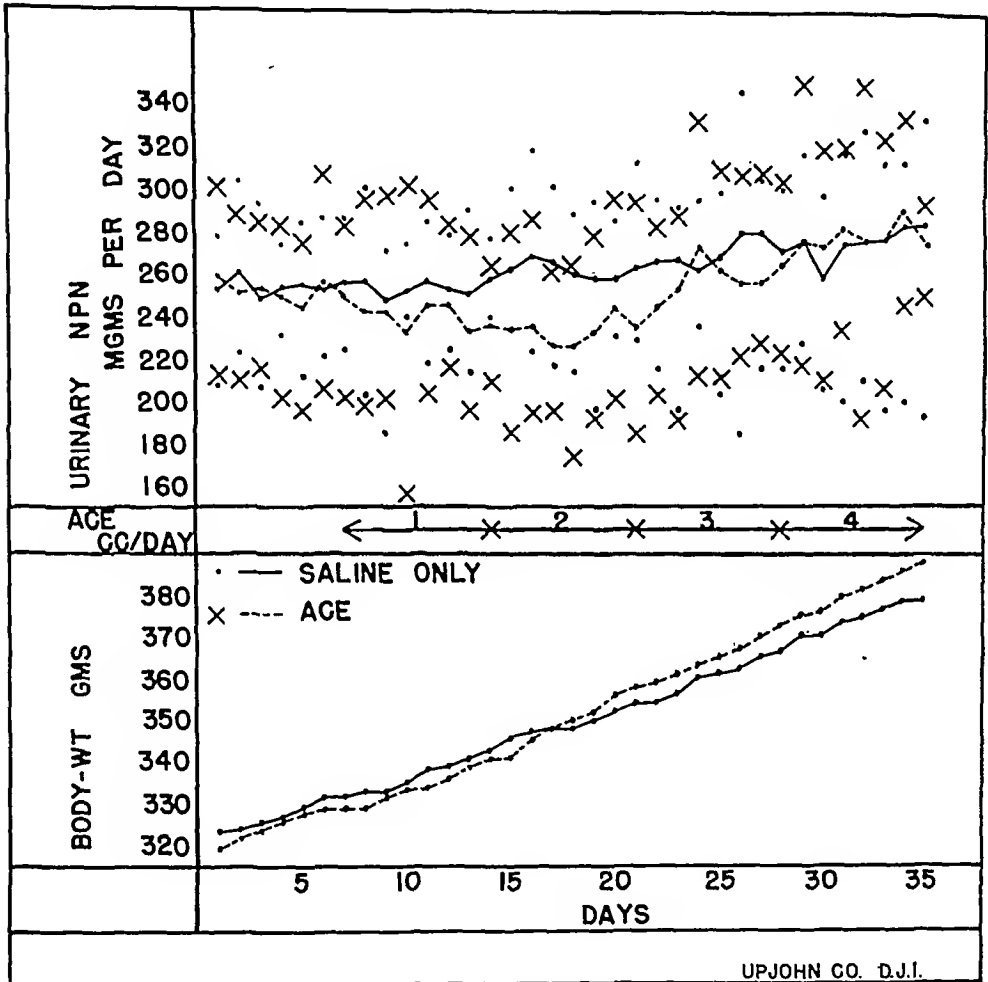


FIG. 1. The effect of adrenal cortex extract upon gain in weight and urinary non-protein nitrogen (averages and range) in adrenalectomized rats. Eleven pairs.

decrease in the excretion of urinary non-protein nitrogen during the administration of 1 and 2 cc. daily of ACE but when the dose was increased to 3 and 4 cc. daily, the level of nitrogen excretion was increased. The group treated with ACE showed greater gains in weight than did the control animals.

In Experiment 2 (Figure 2), 12 adrenalectomized rats were observed during a control period of 14 days. Six of these rats were then treated with 2 cc. of ACE daily for 18 days. The 6 control rats were

given 2 cc. per day of 0.9 per cent sodium chloride solution. There was a prompt decrease in the level of urinary non-protein nitrogen during the administration of ACE and an accompanying increase in the rate of gain in weight.

DISCUSSION

Although the adrenally insufficient rat is capable of growing when maintained on a normal caloric intake and saline to drink, it seems

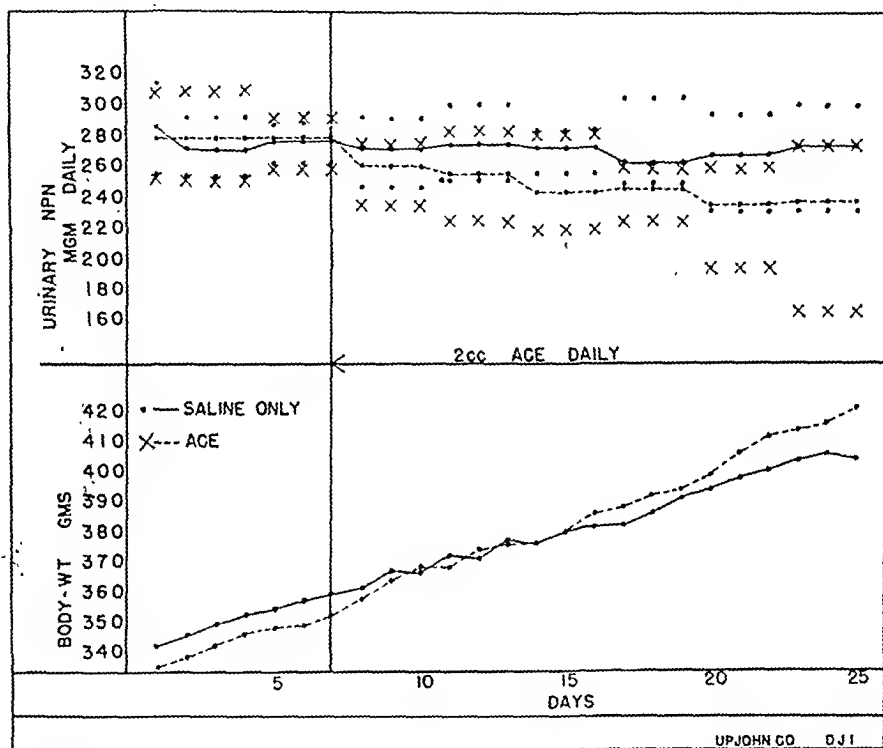


FIG. 2. The effect of adrenal cortex extract upon gain in weight and urinary non-protein nitrogen (averages and range) in adrenalectomized rats. Six pairs.

probable that hormones contained in ACE are essential for the animal to attain an optimal rate of anabolism. A number of other studies (Koelsche and Kendall, 1935; Berman *et al*, 1947; Ingle and Oberle, 1946; Ingle, Ward and Kuizenga, 1947; Ingle and Prestrud 1948) have called attention to experimental conditions in which the adrenal cortical hormones support anabolism. Large amounts of adrenal steroids, especially the 11,17-oxygenated compounds, may cause rapid depletion of some of the body tissues, but it may be correct to characterize these changes as overdosage effects. Several other hormones including thyroxin, androgens and insulin can stimulate catabolism when given in large amounts, although smaller amounts may favor anabolism.

The relationship of the thyroid to growth may be comparable to that of the adrenal cortex. The growth of the thyroidectomized rat is stunted; growth is stimulated by treatment with physiological amounts of thyroxin, but the administration of excessive amounts of this hormone inhibits growth. The changes in nitrogen balance of force-fed thyroidectomized rats parallel the effect of thyroxin upon growth (Rupp and Paschkis, 1948).

Androgens are known to stimulate anabolism. Does ACE contain physiologically important amounts of androgens? The question has not been fully answered but we have never been able to demonstrate an effect of ACE upon the weight of seminal vesicle of the castrate rat.

Ingle (in press) has reviewed the evidence that the relationship of the cortical hormones to growth may be unspecific. It is possible to rationalize at least some of the data by the assumption that the inhibitory effects of the 11-oxygenated adrenal steroids represent overdosage; and by the assumption that the inability of the saline-treated adrenalectomized animal to grow at an optimal rate and its inability to normally accelerate its mobilization of endogenous proteins during stress may each represent a general inability of any metabolic process to reach a maximum response in the absence of the cortical hormones. There may be no complete dropping out of any metabolic process in the adrenally insufficient animal. When conditions of diet and environment are optimal and when the load is light, the rates of many processes approximate those of normal animals at rest but the adrenally insufficient organism is incapable of maximal acceleration of the process under stimulation.

A second hypothesis holds that the hormones of the adrenal cortex such as the 11-oxygenated compounds represent specific growth inhibitors which are normally balanced against the growth hormone of the anterior pituitary. To support this concept (Baker *et al.* 1948) are the many observations that the adrenal cortical steroids inhibit growth and that following adrenalectomy there is increased growth of lymphoid tissue (Reinhardt and Holmes, 1940), hair (Butcher, 1941), and an increase in the growth of bone (Wyman and Tumsuden, 1945).

Elucidation of the relationship of the adrenal hormones to growth will require further study of the effects of the specific adrenal steroids upon specific tissues.

SUMMARY

Two experiments were carried out upon force-fed adrenalectomized male rats having initial weights of approximately 300 grams. In Experiment 1, 11 adrenalectomized rats were given injections of 1 cc. of adrenal cortex extract for one week, 2 cc. for one week, 3 cc. for one week, and 4 cc. for one week. An equal number of control

animals were given injections of physiological saline. The level of urinary NPN was decreased and the rate of weight gain was accelerated by 1 and 2 cc. of ACE per day but when the dose was increased to 3 and 4 cc. the level of nitrogen excretion was increased.

In Experiment 2, 6 adrenalectomized rats were given 2 cc. of ACE daily for 18 days. There was a decrease in the level of urinary NPN and an increase in the rate of weight gain above that of the 6 control animals.

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EFFECTS OF UNDERFEEDING ON THIOURACIL ACTION IN RATS AND MICE

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THE ABILITY of thiouracil to induce thyroid hypertrophy is believed to be mediated through a direct depression of thyroid hormone synthesis, thereby permitting increased thyrotrophic hormone production by the anterior pituitary and greater stimulation of the thyroid (MacKenzie and MacKenzie, 1943; Astwood *et al.*, 1943). Underfeeding is believed to inhibit thyroid function through a direct depression of thyrotrophic hormone secretion by the anterior pituitary (Mulinos and Pomerantz, 1940, 1941; Stephens, 1940; and others). Thiouracil action therefore might be expected to be less effective in underfed than in normally-fed animals, since the initial thyroid and thyrotrophic hormone secretion rates would be lower than in normally-fed animals.

In a previous report on the effects of starvation on thiouracil action in rats, Gomez Mont, Paschkis and Cantarow (1947) found that the hypertrophy and hyperplasia of the thyroids was less marked and developed later than in normally-fed controls. These workers administered the thiouracil as a 0.05 per cent solution to which the underfed and normally-fed rats had free access. It seems doubtful that by this procedure the underfed rats would have consumed as much of the thiouracil solution as the normally-fed rats, since it is well known that starved animals usually reduce their fluid intake. The senior author (J. M.) has repeatedly observed that in a temperature and humidity-controlled animal room, water intake of rats and mice is more or less proportional to feed intake. It seems possible, therefore, that a reduced thiouracil intake by the underfed rats could account for the results reported by Gomez Mont *et al.* (1947).

In the experiments reported here, the effects of both a constant injected dose and of various levels of orally administered thiouracil were determined in rats and mice on restricted diets. The results indicate that on a 100 gm. body weight basis, the underfed animals which received the same dose of injected thiouracil showed the same increase in thyroid weight as the *ad libitum*-fed controls, while the

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animals which were fed less thiouracil than the controls showed a reduced thyroid response.

METHODS

A total of fifty-eight mature female albino rats of the Michigan State College strain and fifty-nine immature and mature male albino mice of the Rockland strain were used in these experiments. The rats and mice were each divided into groups which received the same dose of thiouracil by injection, or different amounts of feed containing thiouracil.

In the rats, thiouracil administration was started simultaneously with underfeeding, and continued for ten days only. The injected rats received an aqueous suspension of ten mg. of thiouracil daily, given in two divided, subcutaneous injections. The thiouracil-fed rats received the drug in the form of a 0.1 per cent mixture in the feed. The restricted diet rats were given either $\frac{3}{4}$ or $\frac{1}{2}$ of the feed consumed daily by the *ad libitum*-fed controls. On the 11th day, all the rats were sacrificed, and the thyroids were carefully removed and weighed on a Roller-Smith balance.

The mice were similarly put on *ad libitum*, $\frac{3}{4}$ or $\frac{1}{2}$ normal feed regimens, but this was continued for 24 instead of ten days. From the 15th through the 24th day, each mouse was injected subcutaneously, once daily, with an aqueous suspension of 10 mg. of thiouracil, or was given feed containing 0.2 per cent thiouracil. The mice were sacrificed on the 25th day. The thyroid weights of the mice and rats were all subjected to statistical analysis. All animals were maintained in a temperature controlled room at 75°F.

RESULTS

Rats

The data on the rats are summarized in Table 1. It can be seen that the control rats (group 1), fed a thiouracil-free diet *ad libitum*, had thyroids weighing 8.1 ± 0.6 mg. per 100 gm. body weight, while the rats fed the 0.1 per cent thiouracil diet *ad libitum* (group 2) had thyroids weighing 24.4 ± 1.5 mg. per 100 gm. body weight. Groups 3 and 4, which were on $\frac{3}{4}$ and $\frac{1}{2}$ normal feed intakes, and consequently ate only $\frac{3}{4}$ and $\frac{1}{2}$ of the thiouracil consumed by group 2, had thyroids which were significantly smaller in size than the rats of group 2 on a 100 grams body weight basis. Groups 5, 6 and 7, which received the same amount of thiouracil by injection, all showed similar increases in thyroid weight on a 100 gm. body weight basis, regardless of the differences in feed consumption.

Mice

The data on the mice are summarized in Table 2. The thiouracil-fed mature mice will be considered first. The mice of group 1, which were permitted to eat *ad libitum* a diet free of thiouracil, had thyroids weighing 8.1 ± 0.9 mg. per 100 gm. body weight, while the mice of group 2, which were fed *ad libitum* a diet containing 0.2 per cent thiouracil, had thyroids weighing 37.3 ± 1.5 mg. per 100 gm. body weight. The mice of group 3, which received only one-half the amount of feed containing the 0.2 per cent thiouracil consumed by

TABLE 1. EFFECTS OF THIOURACIL ON THYROID OF UNDERFED FEMALE RATS

Thiouracil fed							
Group	No. rats per group	Procedure	Orig. body wt. gm.	Final body wt. gm.	Diff. in body wt. %	Thyroid wt. mg.	Thyroid wt. 100 gm. body wt. mg.
1	10	Fed <i>ad lib.</i> 10 days; no thiouracil in feed	145.0	165.0	+13.8	14.0	8.1±0.6*
2	8	Fed <i>ad lib.</i> 10 days; 0.1% thiouracil in feed	145.5	143.5	- 1.3	35.0	24.4±1.5†
3	8	Fed $\frac{3}{4}$ <i>ad lib.</i> 10 days; 0.1% thiouracil in feed	147.5	128.4	-12.9	26.7	20.8±1.4†
4	8	Fed $\frac{1}{2}$ <i>ad lib.</i> 10 days; 0.1% thiouracil in feed	145.5	119.8	-17.6	23.0	19.1±1.1†
Thiouracil injected							
5	8	Fed <i>ad lib.</i> 10 days; 10 mg. thiouracil injected daily	155.0	162.0	+ 4.5	34.3	21.2±1.2
6	8	Fed $\frac{3}{4}$ <i>ad lib.</i> 10 days; 10 mg. thiouracil injected daily	158.0	149.2	- 6.2	32.1	21.5±1.3
7	8	Fed $\frac{1}{2}$ <i>ad lib.</i> 10 days; 10 mg. thiouracil injected daily	157.0	131.0	-15.9	28.8	21.9±1.6

* Standard Error of Mean.

† The means from group 3 and 4 are significantly less than the mean for group 2.

TABLE 2. EFFECTS OF THIOURACIL ON THYROIDS OF UNDERFED MALE MICE

Thiouracil fed							
Group	No. mice per group	Procedure	Orig. body wt. gm.	Final body wt. gm.	Diff. in body wt. %	Thyroid wt. mg.	Thyroid wt. 100 gm. body wt. mg.
1	8	Fed <i>ad lib.</i> 24 days; no thiouracil in feed	32.8	35.5	+ 8.2	2.86	8.1±0.9*
2	8	Fed <i>ad lib.</i> 24 days; 0.2% thiouracil in feed last 10 days	32.0	36.3	+ 13.4	13.62	37.3±1.5
3	8	Fed $\frac{1}{2}$ <i>ad lib.</i> 24 days; 0.2% thiouracil in feed last 10 days	31.2	25.2	- 19.2	4.16	17.0±1.7
Thiouracil injected							
4	10	Fed <i>ad lib.</i> 24 days; no thiouracil injected	9.5	28.3	+197.9	3.11	11.0±0.7
5	7	Fed <i>ad lib.</i> 24 days; 10 mg. thiouracil injected daily last 10 days	9.1	26.7	+193.4	7.98	30.0±5.6
6	10	Fed $\frac{3}{4}$ <i>ad lib.</i> 24 days; 10 mg. thiouracil injected daily last 10 days	10.5	22.2	+111.4	5.79	26.1±2.1
7	8	Fed $\frac{1}{2}$ <i>ad lib.</i> 24 days; 10 mg. thiouracil injected daily last 10 days	10.3	15.8	+ 53.3	5.53	35.0±2.9

* Standard Error of Mean.

group 2, had thyroids which weighed only 17.0 ± 1.7 mg. per 100 gm. body weight, or about one-half the weight of the thyroids of group 2.

The underfed immature mice of groups 6 and 7, which were all injected with the same dosage of thiouracil, showed the same increases in thyroid weight per 100 gm. of body weight as the *ad libitum*-fed controls (group 5). The effects of restricted feed intake on the body weights of these mice was more severe than in the mature mice and rats.

DISCUSSION

The data presented here indicate that underfeeding does not reduce the response of the thyroids to a constant dosage of thiouracil in underfed rats and mice on a body weight basis. The opposite findings of Gomez Mont *et al.* (1947) in underfed rats may well have been due to a lowered thiouracil intake, which was shown here to elicit a smaller response in both rats and mice.

These data do not indicate that underfeeding increases the sensitivity of the thyroids of rats and mice to thyrotrophic hormone, although such a possibility cannot be completely excluded. Stephens (1940) found that the thyroids of starved guinea pigs were more reactive to injected thyrotrophic hormone than normally-fed guinea pigs, but Mulinos and Pomerantz (1941) were unable to corroborate this observation in underfed rats.

The fact that thiouracil can still induce an increase in thyroid size during even severe dietary restriction, as was the case in the mice given one-half of the normal feed intake, indicates that the thyroid gland does not completely cease functioning during starvation. If the thyroids had stopped secreting thyroid hormone altogether, then thiouracil would obviously have had no effect.

The similar increases in thyroid weight per unit of body weight observed in both rats and mice given the same dosage of thiouracil, suggests that thyroid secretion in underfed animals is reduced only in proportion to loss in body weight. Further confirmation of this idea comes from the observation that in intact mature female rats which received either $\frac{3}{4}$, $\frac{1}{2}$ or $\frac{1}{4}$ of the feed consumed by *ad libitum*-fed controls for fourteen days, or no feed for seven days, the weight of the thyroid per unit of body weight remained the same in each case (Meites and Reed, 1949). The thyroidal status during undernutrition, therefore, differs from gonadal and adrenal cortical status, since the gonads may stop functioning entirely while the adrenal cortex usually becomes more active during severe underfeeding.

SUMMARY

The influence of underfeeding on the response of the thyroid gland to either constant or varied amounts of thionracil was de-

terminated in fifty-eight mature female rats and in fifty-nine mature and immature male mice. Thiouracil was injected subcutaneously in the form of an aqueous suspension at the rate of 10 mg. daily or was fed in the ration as a 0.1 or 0.2 per cent mixture. Food intake was restricted by feeding either $\frac{3}{4}$ or $\frac{1}{2}$ of the feed consumed daily by *ad libitum*-fed controls.

It was found that on a 100 gm. body weight basis, the underfed and *ad libitum*-fed animals which received the same amount of thiouracil showed the same increases in thyroid weight, while the animals which received less thiouracil showed reduced thyroid responses. These results suggest that thyroid secretion in underfed rats and mice is reduced only in proportion to loss in body weight.

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INFLUENCE OF THE DIETARY PROTEIN CONCENTRATION UPON THE CORTICOTROPHIC ACTION OF LYOPHILIZED ANTERIOR PITUITARY

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IT HAS BEEN previously reported that the production of nephrosclerosis and hypertension by lyophilized anterior pituitary (LAP) is particularly pronounced on high-protein diets (30%) (Dontigny, Hay, Prado and Selye, 1948; Hay, Prado and Selye, 1948). Further experiments with protein hydrolysates (DeGrandpré, Prado, Dontigny, Leduc and Selye, 1948) and amino-acids (Henriques, Henriques, De Grandpré and Selye, 1948) showed that the latter are responsible for this sensitizing effect of protein. In all these experiments, a marked adrenal enlargement, nephrosclerosis and hypertension were observed in animals treated with LAP and kept on high-protein diets, while slight stimulation of the adrenals, no nephrosclerosis and a normal blood pressure were recorded in similarly treated rats receiving low-protein diets. It was suggested that the hypertension produced by LAP is mediated through the adrenals and kidneys and that the corticotrophic action of hypophyseal extracts is enhanced by dietary protein (Selye, 1946, 1948).

Conversely, the development of hypertension and nephrosclerosis in animals with an "endocrine kidney" (Selye and Stone, 1948) and in those treated with DCA (Prado, Dontigny and Selye, 1947), is not influenced by the protein content of the diet.

These findings suggested that the site of action of dietary protein is between the pituitary and the adrenal cortex; it either increases the efficacy of the corticotrophic hormone (ACTH) or stimulates ACTH production.

It was reported that the ACTH activity of pure preparations is independent of dietary proteins (Ingle, Prestrud, Li and Evans, 1947; Moya, Prado, Rodriguez, Savard and Selye, 1948). Moya *et al.* (1948) showed that under stress ("alarm reaction") the ACTH production, as detected by adrenal ascorbic acid depletion, is increased by diets rich (30%) in protein. It could be that during stress, in addition to ACTH, other corticotrophic pituitary constituents are

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formed and that these are activated by protein. Alternately, protein could augment ACTH-secretion.

In order to gain further information concerning this point, we have studied the effect of dietary protein upon the corticotrophic activity of a total anterior-pituitary preparation (LAP) after hypophysectomy. We used adrenal ascorbic-acid depletion (experiment I) and adrenal enlargement (experiment II) as criteria.

I. INFLUENCE OF DIET ON ASCORBIC ACID DEPLETION ACTIVITY OF LAP

Material and Methods.—Male piebald rats, weighing 120–140 gm. were divided into 2 groups and fed on experimental diets (see table 1) for 15 days. Group I received a 5% casein-diet (Diet 1) and group II a 30% casein diet (Diet 3). At the end of this period, all rats were hypophysectomized and 21

TABLE 1. COMPOSITION OF DIETS

Diets	1	2	3
Casein	5	10	30
Starch	88	83	63
Fat	1	1	1
Steenbock's salt mixture	4	4	4
Cod liver oil	1	1	1
Cellu flour	1	1	1
Vitamins*	1 ml.	1 ml.	1 ml.

* Hawk, Oser and Summerson, 1947.

to 27 hours later the corticotrophic activity of LAP was determined by the ascorbic acid depletion method, following exactly the experimental design and schedule recommended by Sayers, Sayers and Woodbury (1948). 2.6 mg. per 100 gm. of body weight of a fresh 1% solution of nembutal was used as anesthetic. For the adrenal ascorbic acid determinations, the method of Roe and Kuether (1943) was employed. The LAP was ground, passed through a no. 18 U. S. standard sieve (1 mm. opening) and carefully homogenized. 100 mg. of LAP were thoroughly extracted with 25 ml. of 0.01N NaOH in 0.9% NaCl and the solution made up to 100 ml. with 0.9% NaCl (stock solution). The solutions used for injection were prepared by appropriate dilution of the stock solution. For each test a new stock solution was prepared. Several preliminary tests were made to obtain the useful dose-range.

The final assay was performed with the doses 3.5, 7 and 14 γ of LAP per 100 gm. of body weight, given by intra-jugular injection.

RESULTS

Chart 1 and table 2 show the dose-response relationship in both groups of animals, as judged by the resulting adrenal ascorbic acid depletion. The data were submitted to variance analysis (table 3) according to Bliss and Marks (1939), the significance of differences of variances being tested through Fisher's z test (Fisher, 1944). The item "samples" in table 3 indicates the variance produced by the use of two different groups of animals, i.e., animals treated with 5%

and 30% casein. It should be observed that the variance produced by "samples" is smaller than the experimental error; therefore an increase of 5% to 30% in dietary protein concentration caused no statistically significant change in the sensitivity of rats to the corticotrophic activity of LAP as observed by the ascorbic acid depletion test. This is further substantiated by computing the ratio of corticotrophic activity of LAP in both groups of animals. The potency

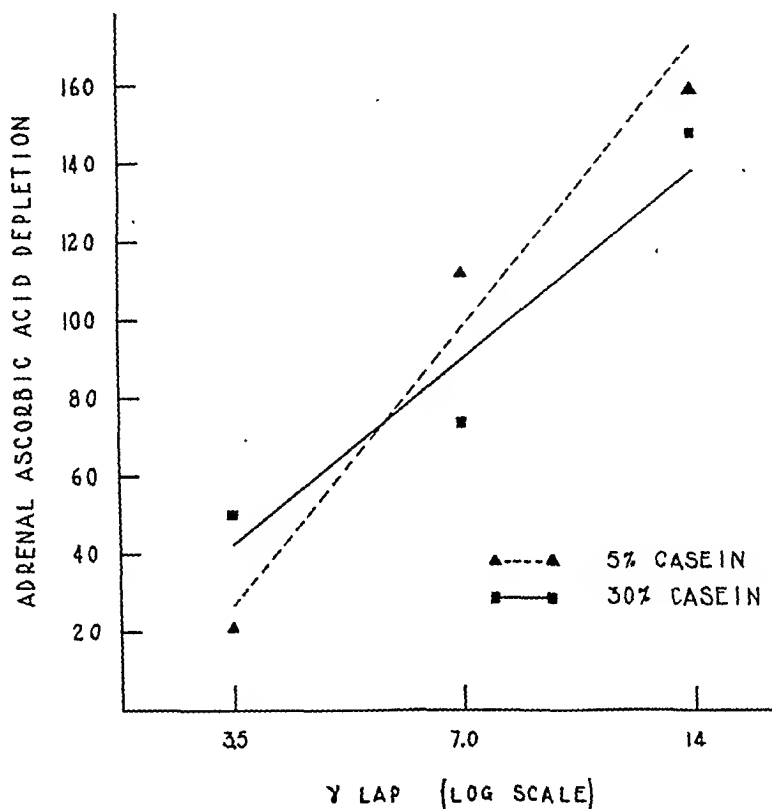


CHART I

ratio obtained was 1.08 ± 0.39 in favor of the animals treated with 5% casein, but this ratio is not significantly different from 1.

11. INFLUENCE OF DIETARY PROTEIN ON ADRENAL WEIGHT RESPONSE TO LAP

Material and Methods.—Two groups of rats whose body weight ranged between 90–110 gm. were adapted to a 10% casein diet (diet 2) for a period of ten days. Group I was then hypophysectomized and was given LAP in two subcutaneous injections of 0.2 ml. per day, increasing progressively from 2 mg. on the first day, to 8 mg. on the seventh. Group III was maintained intact and received the same treatment as group I. Two other similar groups

TABLE 2. INFLUENCE OF DIET ON ADRENAL ASCORBIC ACID RESPONSE TO LAP IN HYPOPHYSECTOMIZED RATS

Group	Treatment	LAP dose (/100 gm. B.W.)	Ascorbic acid depletion
I	5% Casein	3.5	21*
		7.0	111
		14.0	156
II	30% Casein	3.5	50
		7.0	73
		14.0	145

* Each value represents the average of 2 animals.

(II and IV) of rats were adapted for ten days to a 30% casein diet and were treated as groups I and III respectively. All animals received a 5% glucose solution as drinking fluid after the hypophysectomy. At the end of seven days the animals were killed and the completeness of the hypophysectomy checked. The weight of the adrenals was determined after fixation in formalin. The differences in final body weight due to the diets required an exact statistical analysis of the results. It is customary to express the adrenal weight per 100 gm. of body weight as obtained by straight division of the latter by

TABLE 3. VARIANCE ANALYSIS OF ASCORBIC ACID DEPLETION TEST

Variance due to	Sums of squares	n	Variance	Z	P
Samples	126.75	1	126.75	1.12	<0.05
Slope	26680.50	1	26680.50		
Departure from parallelism	861.12	1	861.12		
Curvature of combined curve	4.17	1	4.17	0.428	>0.05
Opposed curvature of separate curves	1441.50	1	1441.50		
Repetitions	6674.16	1	6674.16		
Error	11320.79	4	2830.20		

the former. Korenchevsky (1942) showed that this relative value diminishes with increasing size of the animal. We therefore decided to correct for differences in the average body weights of the various groups by employing a body-adrenal weight regression line obtained through covariance analysis (Bliss and Marks, 1939, 1939a).

RESULTS

As may be seen from table 4, in the hypophysectomized rats there is no significant difference between the average adrenal weights of groups I (10% casein) and II (30% casein) either as absolute weights or corrected for body weight differences ($t=0.956$, $P \approx 0.3$ and $t=1.806$, $P \approx 0.1$ respectively). On the other hand, among the intact animals there is a significant difference between the average adrenal weights of groups III (10% casein) and IV (30% casein) expressed either way. (For absolute weights $t=3.986$, $P < 0.01$ and for corrected weights $t=3.750$, $P < 0.01$).

TABLE 4. INFLUENCE OF DIET ON THE ADRENAL WEIGHT RESPONSE TO LAP IN HYPOPHYSECTOMIZED AND INTACT RATS

Group	No. of animals	Treatment	Body weights		Adrenal weight	
			Initial	Final	mg.	Corrected for B.W.*
I	6	Hypo-ect* 10% casein + LAP	117.5 ± 5.9†	103 ± 16.3†	18.3 ± 0.62†	18.6 ± 0.81
II	6	Hypo-ect 30% casein + LAP	120 ± 5.2	116 ± 17.3	20.0 ± 1.02	19.7 ± 0.81
III	8	10% casein + LAP	116 ± 3.2	130 ± 12.7	23.8 ± 1.02	24.1 ± 0.96
IV	8	30% casein + LAP	120 ± 2.1	148 ± 6.6	29.9 ± 1.15	29.2 ± 0.96

* Hypo-ect means hypophysectomy

† Standard deviation.

‡ Standard error.

§ The correction was made by covariance analysis (Bliss and Marks, 1939, 1939a).

Incidentally, we should like to mention that even on very low protein diets, stress can increase the pituitary ACTH secretion to some extent. This is revealed by the changes in ascorbic acid concentration in the left adrenal of the hypophysectomized rats, that is, the gland removed before LAP injection. It is observed that the adrenal ascorbic acid concentration was consistently and significantly lower on 5% than on 30% casein diets. This fact suggests that the animals on 5% casein secreted an increased amount of ACTH previous to the hypophysectomy. Here the stress effect of the protein deficient diet presumably outweighed its power to inhibit corticotrophin secretion.

TABLE 5. INFLUENCE OF DIETARY PROTEIN ON ADRENAL ASCORBIC ACID CONCENTRATION

Tests	Adrenal ascorbic acid (mg./100 gm)*	
	5% casein	30% casein
1	349 ± 14.1† (7)	432 ± 16.7 (8)
2	352 ± 7.8 (6)	424 ± 20.2 (7)
3	448 ± 4.6 (6)	586 ± 41.7 (6)
4	454 ± 7.0 (6)	526 ± 11.8 (8)

* Control gland removed before injection.

† Standard errors.

Figures between brackets indicate number of animals.

Table 5 summarizes pertinent data obtained in three preliminary experiments (not reported here in detail) as well as in the ascorbic acid depletion test described above.

DISCUSSION

It is apparent that among intact animals, the adrenal enlarging activity of LAP is increased by a high-protein diet, but this is not so in hypophysectomized rats. The latter was observed in both ascorbic acid depletion and adrenal enlargement tests. Our results seem to exclude the possibility of an extra-hypophyseal activation of corticotrophic action by protein. But the possibility still remains that in intact animals under stress, the high-protein diet stimulates the elaboration by the pituitary of corticotrophic material. LAP could

also stimulate the hypophysis of intact animals through stress caused by its toxic components. It should be noted that in spite of LAP treatment, the hypophysectomized animals in experiment II did not gain weight during the week following operation, indicating inadequate food intake. The animals treated with 10% casein lost weight, while those on 30%, roughly maintained their body weights.

The possibility also exists that although protein does not activate LAP directly, it facilitates the formation of active corticotrophin from other LAP-constituents (precursors?) in the pituitary itself.

SUMMARY

Contrary to what is observed in intact rats, after hypophysectomy the corticotrophic action of lyophilized anterior pituitary (LAP) is not influenced by the dietary protein. This was shown both by the adrenal ascorbic-acid-depletion test and by the adrenal weight response of hypophysectomized rats given low and high-protein diets respectively. It appears that the ability of high-protein diets to increase the corticotrophic effect (of stress or LAP) is dependent upon the integrity of the hypophysis.

ACKNOWLEDGMENTS

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EFFECTS OF A HIGH PROTEIN DIET ON THE ANEMIA INDUCED BY HYPOPHYSECTOMY IN ADULT FEMALE RATS, INCLUDING FURTHER DETAILS ON POST-HYPOPHYSECTOMY ANEMIA¹

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HYPOPHYSECTOMY induces a profound anemia in the rat (Stewart, Greep, and Meyer, 1935; Meyer, Stewart, Thewlis, and Rusch, 1937; Vollmer, Gordon, Levenstein, and Charipper, 1939; Crafts, 1941). Injections of thyroxine (Meyer, Thewlis, and Rusch, 1940; Vollmer, Gordon, and Charipper, 1942; Crafts 1946a), a combination of thyroxine, iron, and copper (Crafts, 1946a), and of androgen (Vollmer and Gordon, 1941; Vollmer, Gordon, and Charipper, 1942; Crafts, 1946b) prevented all aspects of this anemia except for the decrease in hemoglobin values. Since the above treatments did not produce a normal hemoglobin, the metabolism of iron was investigated following hypophysectomy. A decrease in volume of gastric juice and a decrease in serum iron was found following hypophysectomy in adult female rats, but the concentration of storage iron was increased (Crafts and Walker, 1947a and b). Utilizing the isotope technique, it was found that there were differences in the metabolism of iron between normal and hypophysectomized rats but that these differences were not of a degree to account for the lack of hemoglobin formation (Crafts, Ross, and Walker, in press).

The present investigation was done in an attempt to answer the question whether the decrease in hemoglobin formation following hypophysectomy is due to a lack of protein to combine with the available iron. Fifty-seven hypophysectomized adult female rats were divided into the following five groups and given the following treatments:—(1) no treatment, (2) a high protein diet, (3) a high protein diet plus daily injections of thyroxine, (4) a high protein diet plus daily injections of testosterone propionate, and (5) a high protein diet plus daily injections of both thyroxine and testosterone propionate.

METHODS

Adult female rats of the Long-Evans strain were used in these experiments. The average body weight of these animals was 196 (S.D. 16.0) gm.

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² With the technical assistance of Miss Shirley A. Raymond.

Hypophysectomy was performed from the parapharyngeal approach and the completeness of the operation was checked by serial sections of the hypophyseal capsule.

Because no studies were contemplated on the white blood cells, blood for these studies was obtained by clipping the tail. Absolutely no "milking" of the tail was performed. Five to six drops of free flowing blood were placed in a small vial containing powdered heparin.

Erythrocyte counts were made in duplicate using standard U. S. certified pipettes and the improved Neubauer counting chamber. Hemoglobin determinations were made on an Evelyn photoelectric colorimeter. Van Allen tubes were used for hematocrit determinations. Mean corpuscular diameter (M.C.D.) was measured in a Haden-Hausser erythrocytometer using cover slip smears stained with Wright's stain and mounted in physiological saline solution. Mean corpuscular volume in cubic microns (M.C.V.), mean corpuscular hemoglobin in micromicrograms (M.C.H.), and mean corpuscular hemoglobin concentration in per cent (M.C.H.C.) were determined by the formulae cited below.³

The high protein diet was obtained by combining the standard diet (Rockland Farms complete rat diet) with powdered casein in equal quantities. This produced a diet containing 62.5% protein. Fresh lettuce was given weekly.

PROCEDURE AND RESULTS

Table 1 shows the average figures obtained for the 57 rats used in these experiments before any treatment. The erythrocyte count averaged 7.83 million cells per cu. mm.; hematocrit, 43.1%; hemoglobin, 14.0 gm. per 100 cc.; M.C.D., 5.63 micra; M.C.V., 55.2 cu. microns; M.C.H., 18.0 micromicrograms; and the M.C.H.C., 32.5%. These figures for the erythrocyte count, hemoglobin, and the diameter closely agree with figures previously published from this laboratory. The hematocrit (43.1%) is slightly lower than the average of 45.05% found by Gardner (1947) in summarizing values in the literature. Her average, however, includes both male and female rats.

Hypophysectomy

Hypophysectomy induced an anemia. Much data have been published on the blood picture during the first 40 days after hypophysectomy. Accordingly, these rats were studied from the 40th to the 95th day after removal of the hypophysis. Hypophysectomy induced a decrease in the erythrocyte count (Table 1, Fig. 1) to between 5 and 6 million cells per cu. mm. The counts reached that

$$^3 \text{ M.C.V.} = \frac{\text{vol. packed red cells, cc. per 1000 cc.}}{\text{red cell count, millions per c.mm.}}$$

$$\text{M.C.H.} = \frac{\text{hemoglobin, gm. per 1000 cc.}}{\text{red cell count, millions per c.mm.}}$$

$$\text{M.C.H.C.} = \frac{\text{hemoglobin, gm. per 100 cc.} \times 100}{\text{vol. packed red cells, cc. per 100 cc.}}$$

TABLE 1. EFFECTS OF HYPOPHYSECTOMY, TREATMENT WITH A HIGH PROTEIN DIET ALONE, AND TREATMENT WITH A HIGH PROTEIN DIET PLUS INJECTIONS OF THYRONINE AND TESTOSTERONE PROPIONATE ON THE BLOOD PICTURE OF HYPOPHYSECTOMIZED ADULT FEMALE RATS

DAY = Days of treatment; BW = Body weight; TRC = Total red cell count in millions per cu. mm.; HEMAT = Hematocrit in percent; HB = Hemoglobin in gm. per 100 cc; M.C.D. = Mean corpuscular diameter in micra; M.C.V. = Mean corpuscular volume in cu. microns; M.C.H. = Mean corpuscular hemoglobin in micromicrograms; M.C.H.C. = Mean corpuscular hemoglobin concentration in percent; \pm = Standard deviation.

Normal control figures on all rats before hypophysectomy—57 rats.								
DAY	BW	TRC	HEMAT	HB	M.C.D.	M.C.V.	M.C.H.	M.C.H.C.
0	196	7.83	43.1	14.0	5.63	55.2	18.0	32.5
	± 16	± 0.45	± 1.7	± 0.7	± 0.21	± 2.5	± 0.9	± 1.4
Hypophysectomized—no treatment—5 rats.								
DAY	BW	TRC	HEMAT	HB	M.C.D.	M.C.V.	M.C.H.	M.C.H.C.
40	153	6.20	30.5	10.2	5.52	49.3	16.6	33.6
	± 6	± 0.35	± 0.9	± 0.4	± 0.11	± 2.1	± 0.8	± 0.4
50	159	5.95	30.4	10.3	5.54	51.5	17.4	33.8
	± 15	± 0.53	± 2.1	± 0.5	± 0.13	± 2.3	± 1.3	± 1.4
60	153	5.64	29.5	9.7	5.75	53.0	17.4	33.0
	± 6	± 0.43	± 1.7	± 0.5	± 0.19	± 5.7	± 1.4	± 2.0
75	140	5.19	28.7	9.4	5.55	55.3	18.1	32.7
	± 4	± 0.15	± 1.0	± 0.3	± 0.17	± 1.8	± 0.3	± 0.6
95	132	5.99	29.8	9.9	5.89	50.8	16.5	32.6
	± 14	± 0.37	± 1.3	± 1.0	± 0.23	± 0.9	± 0.8	± 1.8
Hypophysectomized and treated with a high protein diet—8 rats.								
DAY	BW	TRC	HEMAT	HB	M.C.D.	M.C.V.	M.C.H.	M.C.H.C.
0	194	7.79	43.7	13.9	5.81	56.1	17.9	31.9
	± 19	± 0.36	± 1.0	± 0.4	± 0.24	± 1.9	± 0.7	± 0.7
10	178	8.29	45.4	15.1	5.56	54.8	18.2	33.2
	± 15	± 0.64	± 4.1	± 1.3	± 0.08	± 1.3	± 0.8	± 1.3
20	173	8.24	45.3	14.5	5.60	55.1	17.6	31.8
	± 16	± 0.94	± 4.7	± 1.2	± 0.09	± 2.1	± 0.8	± 0.9
30	178	7.14	37.9	12.5	5.67	53.2	17.6	33.2
	± 11	± 0.84	± 3.7	± 1.1	± 0.08	± 4.1	± 0.9	± 1.9
40	183	7.05	35.9	12.1	5.61	51.0	17.2	33.9
	± 10	± 0.24	± 2.9	± 0.5	± 0.11	± 4.7	± 0.8	± 2.4
50	169	6.82	36.8	12.1	5.66	53.8	17.7	32.9
	± 14	± 0.39	± 0.8	± 0.4	± 0.06	± 2.2	± 0.9	± 0.9
Hypophysectomized and treated with a high protein diet plus daily injections of 0.005 mg. of thyroxine. On day 30 the dose of thyroxine was changed to 0.015 mg. per day—14 rats.								
DAY	BW	TRC	HEMAT	HB	M.C.D.	M.C.V.	M.C.H.	M.C.H.C.
0	194	7.90	43.2	13.7	5.65	54.5	17.4	31.8
	± 23	± 0.53	± 1.9	± 0.9	± 0.06	± 2.1	± 0.6	± 0.9
10	179	7.81	42.3	13.8	5.58	53.8	17.8	32.8
	± 21	± 0.46	± 1.7	± 0.5	± 0.13	± 2.5	± 0.8	± 1.0
20	172	7.55	39.7	12.9	5.60	52.5	17.1	32.2
	± 20	± 0.42	± 2.0	± 1.4	± 0.14	± 2.5	± 1.7	± 3.0
30	164	7.33	37.6	12.3	5.61	51.2	16.7	32.7
	± 23	± 0.69	± 3.0	± 1.0	± 0.13	± 2.3	± 0.9	± 0.9
40	154	7.65	39.6	12.8	5.70	52.2	16.8	32.2
	± 24	± 0.81	± 2.6	± 0.9	± 0.13	± 3.3	± 1.2	± 0.8
50	146	7.56	41.1	12.8	5.63	54.4	16.9	31.0
	± 21	± 0.56	± 3.0	± 0.9	± 0.08	± 2.8	± 0.9	± 0.7
60	149	7.65	40.1	12.7	5.80	52.6	16.6	31.6
	± 15	± 0.47	± 1.8	± 0.7	± 0.15	± 2.7	± 1.0	± 1.2

TABLE 1-Cont.

Hypophysectomized and treated with a high protein diet plus daily injections of 2.0 mg. of testosterone propionate—18 rats.

DAY	BW	TRC	HEMAT	HB	M.C.D.	M.C.V.	M.C.H.	M.C.H.C.
0	195	7.67	42.6	13.9	5.72	55.6	18.3	32.6
	±11	±0.41	±1.8	±0.4	±0.13	±2.6	±0.9	±1.6
10	175	7.98	44.3	14.5	5.71	56.4	18.2	32.3
	±16	±0.88	±4.3	±1.4	±0.12	±4.0	±1.3	±1.0
20	172	7.68	42.3	13.7	5.63	55.1	17.9	32.4
	±10	±0.75	±4.1	±1.4	±0.07	±2.4	±0.7	±1.1
30	180	7.36	39.6	12.8	5.75	53.7	17.5	32.2
	±10	±0.24	±1.0	±0.4	±0.13	±2.0	±0.6	±1.2
40	176	7.42	38.4	12.4	5.65	52.1	16.8	32.2
	±9	±0.63	±2.0	±0.5	±0.11	±3.7	±1.6	±1.6
50	176	8.31	39.9	12.9	—	48.4	15.5	32.4
	±11	±0.33	±0.7	±0.4	—	±2.3	±0.8	±1.0

Hypophysectomized and treated with a high protein diet plus daily injections of 0.005 mg. of thyroxine and 1.0 mg. of testosterone propionate. On day 40 all treatment was stopped—12 rats.

DAY	BW	TRC	HEMAT	HB	M.C.D.	M.C.V.	M.C.H.	M.C.H.C.
0	201	7.98	43.3	14.5	5.35	54.2	18.2	33.5
	±16	±0.43	±1.8	±0.6	±0.15	±2.7	±0.9	±0.9
10	181	8.02	43.0	14.1	5.85	53.9	17.7	32.9
	±11	±0.41	±1.4	±0.7	±0.26	±2.2	±0.7	±1.1
20	186	8.43	44.7	14.6	5.56	53.0	17.3	32.6
	±12	±0.23	±1.7	±0.6	±0.24	±2.4	±0.9	±0.7
30	178	8.48	44.2	14.1	5.54	52.0	16.7	32.0
	±11	±0.34	±1.4	±0.7	±0.25	±2.6	±1.1	±0.8
40	173	8.42	44.0	13.9	5.69	52.3	16.5	31.6
	±19	±0.59	±2.9	±0.8	±0.10	±2.6	±0.7	±0.7
All treatment stopped								
10	162	8.48	42.1	13.3	5.85	50.0	15.7	31.5
	±11	±0.65	±2.1	±0.8	±0.08	±2.4	±0.7	±0.8
20	156	7.66	38.4	12.2	5.90	50.2	15.9	31.7
	±16	±0.46	±2.6	±0.6	±0.06	±2.4	±0.6	±1.9
30	152	7.24	34.0	10.7	5.51	47.0	14.8	31.6
	±14	±0.16	±1.8	±0.5	±0.09	±1.7	±0.6	±0.7

level at 50 days and remained at that level for another 45 days. This is a 29.8% decrease. Hematocrit values (Table 1, Fig. 2) decreased to 30.5% on the 40th day after hypophysectomy and remained between 28.7% and 30.5% for the entire time of observation, a 32.6% decrease. Hemoglobin determinations (Table 1, Fig. 3) showed a decrease to 10.2 gm. per 100 cc. on the 40th day after removal of the hypophysis and remained between 9.0 and 10.0 gm. for the remaining 55 days of observation. This is a 31.4% decrease.

The mean corpuscular diameter (M.C.D. Table 1) on the 40th day after hypophysectomy averaged 5.52 micra, a slight decrease from the control average of 5.63 micra. During the subsequent 55 days of observations, while the erythrocyte count, hemoglobin, and hematocrit reached a plateau, the M.C.D. fluctuated. This change is slight compared to the data previously reported (Crafts, 1946a) indicating a more definite microcytosis. This previous work, however, was based on studies at 30 and 40 days after hypophysectomy and on actual measurements with an ocular micrometer.

In spite of the slight decrease in diameter, the mean corpuscular

volume (M.C.V. Table 1) decreased to 49.3 cu. microns (Control 55.2) 40 days after hypophysectomy. Observations during the subsequent 55 days showed a gradual increase to a normal value of 55.3 cu. microns on the 75th day and a drop to 50.8 on the 95th day after removal of the hypophysis.

The mean corpuscular hemoglobin (M.C.H. Table 1) followed the same course as the mean corpuscular volume. At 40 days the M.C.H.

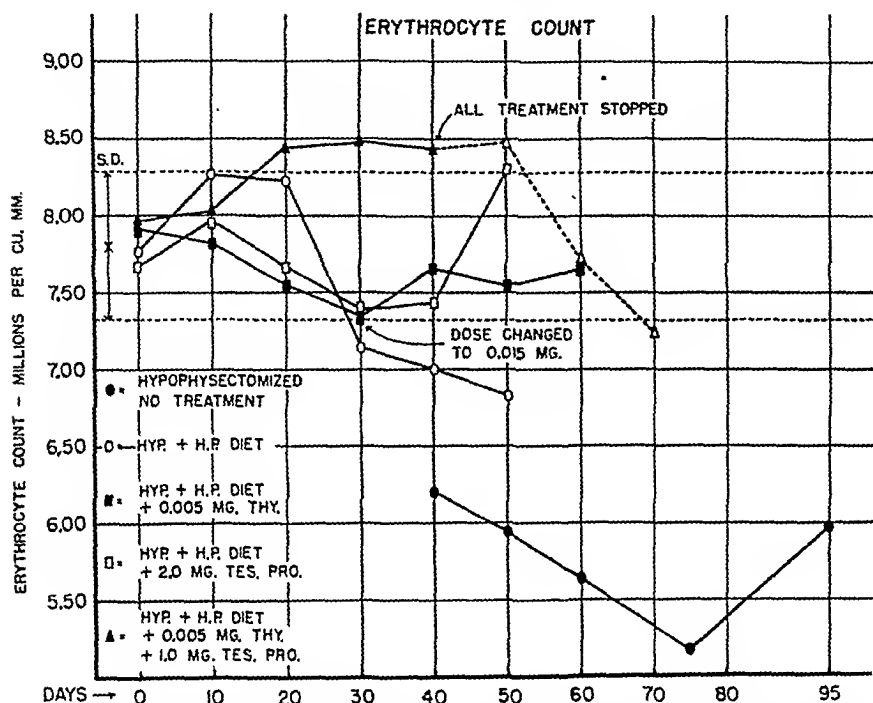


FIG. 1. The effects of a high protein diet, with or without thyroxine or testosterone propionate therapy, on the erythrocyte count of hypophysectomized adult female rats. X-S.D. = mean and standard deviation of all rats before treatment.

was 16.6 micromicrograms (Controls 18.0). The values gradually increased to a normal figure of 18.1 micromicrograms and then dropped to 16.5 on the 95th day after hypophysectomy.

The mean corpuscular hemoglobin concentrations (M.C.H.C. Table 1) showed no change from normal values.

In summary, hypophysectomy induced a profound anemia. The erythrocyte count, hematocrit, and hemoglobin decreased rapidly for 40 days after which no further decrease occurred. From the 40th to the 95th days after hypophysectomy the mean corpuscular diameter, volume, and hemoglobin fluctuated, at one time showing a decrease and at another time showing normal values. The mean corpuscular hemoglobin concentration did not change, indicating that regardless of size of the erythrocytes, each carried a normal concen-

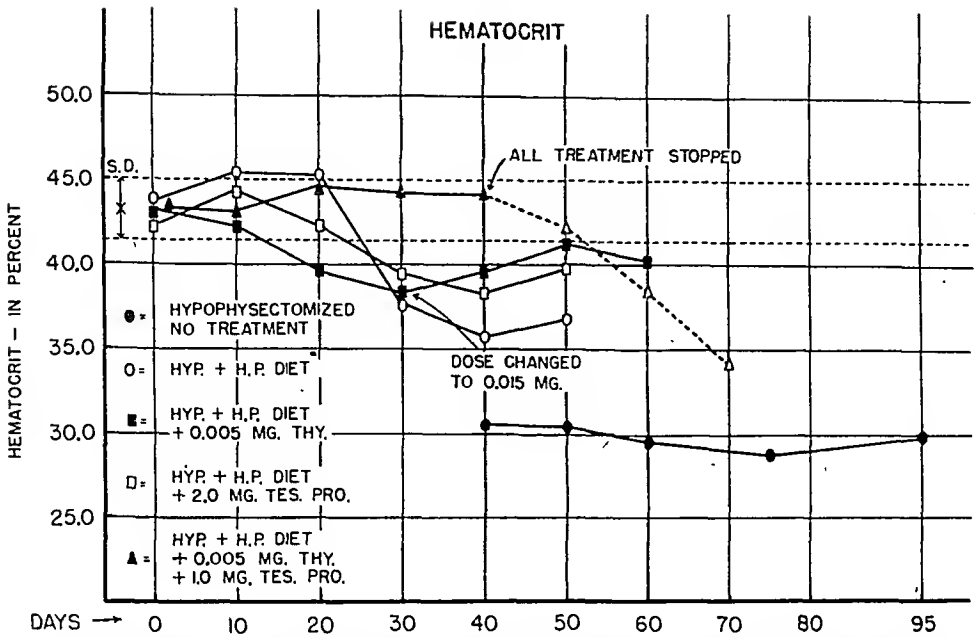


FIG. 2. The effects of a high protein diet, with or without thyroxine or testosterone propionate therapy, on the hematocrit values of hypophysectomized adult female rats. X-S.D. = mean and standard deviation of all rats before treatment.

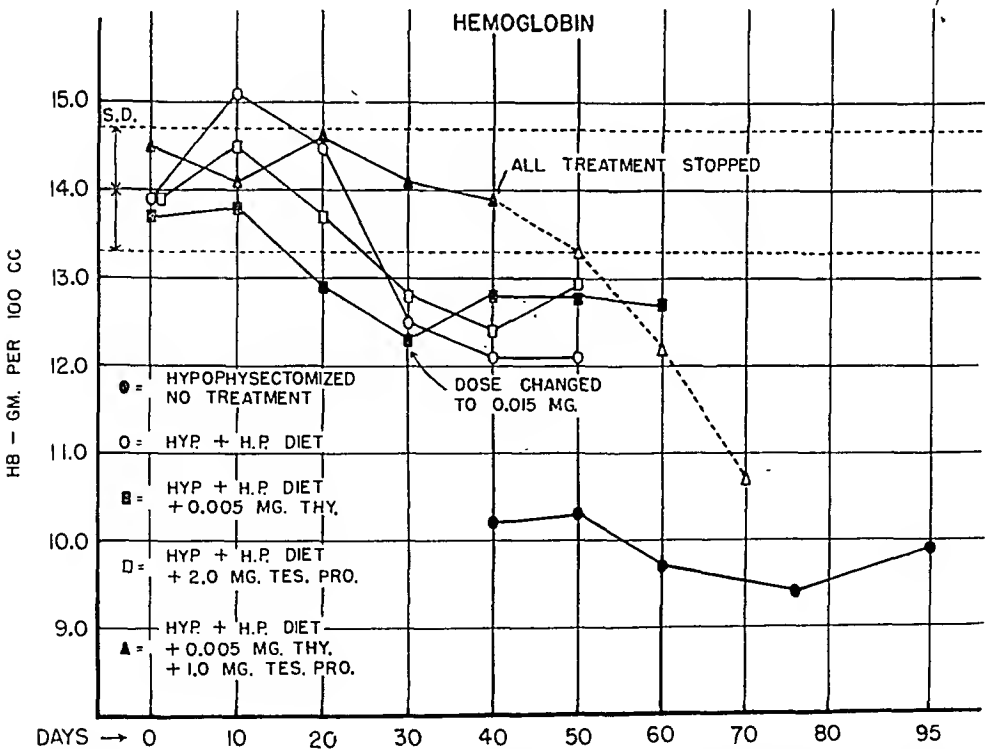


FIG. 3. The effects of a high protein diet, with or without thyroxine or testosterone propionate therapy, on the hemoglobin values of hypophysectomized adult female rats. X-S.D. = mean and standard deviation of all rats before treatment.

tration of hemoglobin. Any decrease in mean corpuscular hemoglobin, therefore, was due to the decrease in volume of the erythrocytes. The hypophysectomized rat finally showed a profound anemia which may be considered as a normocytic normochromic type or a slight microcytic normochromic type. These findings agree with the many reports in the literature for humans with hypopituitary conditions. Some patients show a normocytic normochromic type of anemia while others show a microcytic normochromic type. A single patient may vary from one type to the other at different times just as is indicated in these data for the rat.

High Protein Diet

Fourteen rats were hypophysectomized and immediately placed on a high protein diet. No further treatment was given. Blood was studied at 10 day intervals for 50 days. Detailed results are given in Table 1.

The erythrocyte count (Fig. 1) for this group of rats before hypophysectomy averaged 7.79 million cells per cu. mm. On the 10th day the count had increased to 8.29 million cells, remained at that level on the 20th day, and then gradually decreased to 6.82 million cells 50 days after hypophysectomy. This is a 12.9% decrease.

Hematocrit values followed the same curve (Fig. 2), increasing from the normal level of 43.7% to 45.4 and 45.3% on days 10 and 20 respectively, and then gradually decreasing to 35.9% on day 40 and 36.8% on day 50. This is a 15.8% decrease.

The hemoglobin (Fig. 3) before treatment averaged 13.9 gm. per 100 cc., increased to 15.1 gm. on day 10, and then gradually decreased to 12.1 gm. on days 40 and 50, a 12.9% decrease.

The changes in mean corpuscular diameter, volume, and hemoglobin were slight, probably insignificant decreases, while the M.C.H.C. remained normal.

Summarizing, a high protein diet did not prevent an anemia from developing following the removal of the hypophysis. The anemia, however, was not as severe as is found after hypophysectomy and no treatment. Erythrocyte count, hematocrit, and hemoglobin decreased 13, 16, and 13% respectively, while after hypophysectomy alone there were 30, 33, and 31% decreases in these figures.

High Protein Diet + Thyroxine

Thyroxine therapy has been found to alleviate many of the signs of the anemia induced by hypophysectomy. In addition, moderate doses of thyroxine are known to stimulate the utilization of proteins. Accordingly, 14 hypophysectomized rats were injected with daily doses of 0.005 mg. of thyroxine⁴ in addition to the high protein diet. Treatment was started the day after hypophysectomy. The dose of

⁴ The thyroxine was very kindly furnished by Hoffmann-La Roche, Inc.

thyroxine was elevated to 0.015 mg. on the 30th day. Blood was studied at 10 day intervals for 60 days. Detailed results are shown in Table 1.

The erythrocyte count (Fig. 1) in this group of rats before hypophysectomy averaged 7.90 million cells per cu. mm. There was a decrease to 7.33 million cells by the 30th day but an increase to 7.65 million cells after 30 days of treatment at the high dose. This is only a 3.2% decrease.

The hematocrit (Fig. 2) averaged 43.2% before hypophysectomy. Thirty days after removal of the hypophysis the hematocrit averaged 37.6%, and with the higher dose of thyroxine increased to 40.1% on the 60th day. This is a 7.2% decrease.

Hemoglobin values (Fig. 3) started at an average of 13.7 gm. per 100 cc. and decreased to 12.7 gm. by the 60th day, a 7.7% decrease.

The M.C.V. and M.C.H. showed a slight decrease while the M.C.D. and M.C.H.C. showed no changes.

Summarizing, hypophysectomized rats fed a high protein diet and treated with daily injections of thyroxine for 60 days showed a 3% drop in erythrocyte count, a 7% drop in hematocrit, and an 8% drop in hemoglobin. These decreases are small when compared to a 30% erythrocyte count drop, a 33% hematocrit drop, and a 31% hemoglobin drop which followed hypophysectomy and no treatment.

High Protein Diet + Testosterone Propionate

Testosterone propionate has also been found to alleviate most of the signs of the anemia which is induced by removal of the hypophysis. This hormone is well known to be a stimulant to the utilization of protein in the normal animal and has been reported to have a similar action in the hypophysectomized rat (Kochakian, Moe, Hunter, and Stettner, 1947). Accordingly, 18 hypophysectomized rats were given a high protein diet plus daily injections of 2.0 mg. of testosterone propionate⁵ for 50 days starting the day following the removal of the hypophysis. Blood was studied at 10 day intervals. Details are presented in Table 1.

Erythrocyte values (Fig. 1) started at an average of 7.67 million cells per cu. mm. before treatment. By the 50th day the count had increased to 8.31 million cells, a 7.8% increase rather than a decrease. Hematocrit values (Fig. 2) started at 42.6% and decreased to 39.9%, a 6.3% decrease. Hemoglobin values (Fig. 3) decreased from the normal average of 13.9 gm. per 100 cc. to 12.9 gm., a decrease of 7.2%.

The M.C.V. and M.C.H. decreased from 55.6 to 48.4 cu. microns (13.0% decrease) and from 18.3 to 15.5 micromicrograms (15.3% decrease) respectively. The M.C.D. and M.C.H.C. showed no significant changes.

In summary, testosterone propionate treatment, in addition to

⁵ Testosterone propionate was very kindly furnished by Schering Corporation, Bloomfield, N. J.

the high protein diet, maintained a normal erythrocyte count. Hematocrit and hemoglobin values decreased only 6 and 7% respectively. There was a 13% decrease in M.C.V. and a corresponding 15% decrease in M.C.H.

High Protein Diet + Thyroxine + Testosterone Propionate

Twelve hypophysectomized rats were treated with daily injections of a combination of 0.005 mg. of thyroxine and 1.0 mg. of testosterone propionate in addition to the high protein diet. These rats were studied at 10 day intervals for 40 days. At this time, the 40th day, all treatment was stopped, the animals being placed on the regular diet. Details are presented in Table 1.

The erythrocyte count (Fig. 1) in this group of rats averaged 7.98 million cells per cu. mm. before treatment. The count increased to 8.42 million cells in 40 days of treatment, a 5.2% increase. After treatment was stopped, the count remained at this high level for 10 days and then decreased to 7.24 million cells 30 days after cessation of treatment.

Hematocrit determinations (Fig. 2) increased from 43.3% to 44.0% in 40 days of treatment. Although this is not a significant increase, it is the only treatment in this series of experiments in which no decrease occurred. After treatment was stopped, the hematocrit decreased to 34.0% in 30 days.

The hemoglobin average for this group of rats before treatment was slightly higher than that of the other groups (Fig. 3). This group started at 14.5 gm. per 100 cc. and decreased to 13.9 gm. in 40 days. This figure is a normal average for these rats, for the average of all 57 rats in this series before treatment was only 0.1 gm. higher, i.e. 14.0 (S.D. 0.7) gm. The decrease from 14.5 gm. to 13.9 gm. is a 4.1% drop. After treatment was stopped, the hemoglobin decreased to 10.7 gm. in 30 days, a figure typical of the hypophysectomized animal with no treatment.

The M.C.D. increased slightly and the M.C.V., M.C.H., and the M.C.H.C. declined slightly. After treatment was stopped, the M.C.D. declined, the M.C.V. and M.C.H. decreased even lower, and the M.C.H.C. remained the same.

In summary, hypophysectomized rats, when treated with a high protein diet plus injections of both thyroxine and testosterone propionate, exhibited no anemia. When treatment was stopped, the erythrocyte count, hematocrit, and hemoglobin values decreased. During treatment, the erythrocyte and hematocrit values were increased while the hemoglobin was merely maintained at normal levels. In addition, the M.C.V. declined slightly. As a result the hemoglobin per cell (M.C.H.) decreased slightly and the concentration of hemoglobin in each cell (M.C.H.C.) also decreased slightly. The latter figure, however, is well within the normal range. After treatment was stopped, these effects were accentuated.

DISCUSSION

Adult rats develop an anemia following the removal of the hypophysis. The erythrocyte count exhibits a 30% decrease during the first 40 days after hypophysectomy; the hematocrit, a 33% decrease; and the hemoglobin, a 31% decrease. From the 40th to the 95th day after hypophysectomy the erythrocyte count, hematocrit, and hemoglobin reach a plateau showing no further decreases. The erythrocytes have a decreased mean corpuscular volume and an accompanying decrease in mean corpuscular hemoglobin, although these decreases are small. Each cell, however, carries a normal concentration of hemoglobin which shows that the decrease in mean corpuscular hemoglobin is due to the decrease in size of the erythrocytes. This anemia fluctuates between a normocytic normochromic type and a microcytic normochromic type. The bone marrows are hypoplastic and exhibit a decrease in number of erythroid elements (Crafts, 1946a and b). This anemia is similar to that observed in human hypopituitary patients as reported often in the literature. (See review article by Daughaday, Williams, and Daland, 1948.)

Injections of thyroxine or androgens have been shown to prevent all aspects of this anemia except for the fall in hemoglobin. The data in this report show that the fall in hemoglobin level, in addition to other aspects of the anemia, can be prevented when a high protein diet is added to a combined thyroxine and androgen therapy.

Is the anemia which occurs following the removal of the hypophysis actually due to faulty protein metabolism? Hypophysectomy is well known to interfere with protein metabolism. In addition, Cartwright (1947), in a review article, cites many references showing that an anemia is induced by decreasing the protein content of the diet below normal, and that the formation of hemoglobin is enhanced by a return to a normal or high protein diet. Levin (1943) has shown that the hypophysectomized animal is not able to utilize endogenous protein. On the other hand, if faulty protein metabolism is the partial or complete cause for the anemia which hypophysectomy induces, growth hormone injections should be a logical therapy. As yet, this has not proved to be so. Meyer, Stewart, Thewlis, and Rusch (1937) injected hypophysectomized rats with growth hormone (Antuitrin S—Parke Davis) in the dose of 1 cc. three times a week. They obtained a marked response in reticulocytes but the erythrocyte count and hemoglobin did not rise. When they combined the growth hormone with thyroxine, a good response was obtained. Growth hormone alone, in the doses given and the preparation used, was not effective. No reports have been published on the effects of the purified growth hormone preparations on this anemia.

The anemia induced by hypophysectomy can be prevented by a high protein diet in combination with thyroxine and androgen therapy. The beneficial effects of thyroxine and testosterone propi-

onate may be due to their influence on general metabolism or to their influence on protein metabolism. It is safe to conclude that the anemia that is induced by removal of the hypophysis is due to faulty metabolism and that there is no necessity for a "hemopoietic hormone" secreted by the hypophysis as proposed by Flaks, Himmel, and Zlotnik (1937-1938).

SUMMARY

Hypophysectomy induces an anemia in adult rats which has the following characteristics: (1) a 30% decrease in erythrocyte count, (2) a 33% decrease in hematocrit, (3) a 31% decrease in hemoglobin, (4) a slight decrease in mean corpuscular volume which caused (5) a slight decrease in mean corpuscular hemoglobin, (6) a normal mean corpuscular hemoglobin concentration, and (7) a hypoplasia of the bone marrow with a decrease in percentage of erythroid elements.

Thyroxine or androgen therapy prevent all manifestations of this anemia except for the fall in hemoglobin. This experiment was conducted to determine whether this lack of hemoglobin formation was due to a lack of available protein. 57 hypophysectomized adult female rats were divided into 5 groups: (1) no treatment, (2) fed a high protein diet, (3) a high protein diet + thyroxine, (4) a high protein diet + androgen, and (5) a high protein diet + thyroxine + androgen.

Best results were obtained in the last group with a high protein diet + 0.005 mg. of thyroxine per day + 1.0 mg. of testosterone propionate per day. This therapy maintained the hematocrit and hemoglobin at normal levels while increasing the erythrocyte count. The anemia induced by hypophysectomy can be prevented by a high protein diet in combination with thyroxine and androgen therapy.

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STUDIES ON THE NATURE OF THE PROTEIN CATABOLIC RESPONSE TO ADRENAL CORTICAL EXTRACT. ACCENTUATION BY INSULIN HYPOGLYCEMIA

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IN PREVIOUS REPORTS (Engel, Schiller and Pentz, 1949, Bondy, Engel, and Farrar, 1949) data were presented to indicate that the adrenal cortex influenced nitrogen metabolism largely at the level of protein breakdown and that this effect on protein catabolism was conditioned by the internal metabolic environment of the animal at the time of treatment with hormone. It was suggested that the levels of carbohydrate stores in the body and the availability of sources of new carbohydrate other than tissue protein were factors in determining the occurrence or magnitude of protein catabolism after cortical hormone treatment. A possible synergistic role of non-specific stress in the protein catabolic response to adrenal cortical extract (A.C.E.) was also considered.

The present study is an attempt to elucidate further the role of the need for gluconeogenesis in determining the protein catabolic response to A.C.E. Insulin hypoglycemia was used as a method for increasing carbohydrate requirements and hence the need for gluconeogenesis in the fasting animal. It was found that insulin alone had no immediate effect on the rate of urea accumulation in nephrectomized rats but caused a delayed increase whereas insulin plus A.C.E. resulted in an immediate and striking acceleration of urea formation which was 3-4 times as great as that after A.C.E. alone. Prevention of hypoglycemia by glucose obliterated the increase in nitrogen metabolism.

MATERIALS AND METHODS

These were essentially the same as in previous reports (Engel, Pentz, and Engel, 1948, Engel, Schiller and Pentz, 1949, Bondy, Engel and Farrar, 1949) where full details may be obtained. Use was made of the rate of urea formation in the fasted nephrectomized rat, expressed as mg. urea N/100 grams body weight/hour, as a measure of protein catabolism. The animals

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were nephrectomized 16 hours before the experimental procedure was begun and rates of urea formation studied for successive 3-hour periods. In all cases the first three-hour period was used as a control before test materials other than A.C.E. were injected. Blood urea nitrogen was determined by the method of Archibald, Ortiz, Stroh and Bronner (1945) and blood glucose by the method of Nelson (1944). The xanthidol method for urea (Engel and Engel, 1947) previously used in these studies was not employed because of difficulty in obtaining an adequate grade of xanthidol and subsequent highly irregular results from that material available. Squibb's insulin, diluted with saline and Upjohn's adrenal cortex extract were used throughout and were injected subcutaneously.

TABLE 1. INFLUENCE OF A.C.E. AND INSULIN ON THE UREA PRODUCTION OF NEPHRECTOMIZED RATS

Urea Nitrogen—Mg N per 100 Gm Body Weight per hour								
Procedure	No. of rats	0-3 hours	3-6 hours	Change	P	6-9 hours	Change	P
A.C.E. (1.0 ml per 100 gms)*	10	1.5±0.24†	2.4±0.25	+0.9±0.28	<0.01	—	—	—
Insulin (0.08 unit per 100 gms)†	13	2.3±0.21	3.0±0.35	+0.7±0.41	0.1	4.3±0.28	+2.0±0.23	<0.01
Insulin (0.5 unit per 100 gms)†	11	2.2±0.21	2.5±0.31	+0.3±0.35	>0.4	—	—	—
A.C.E. (1.0 ml per 100 gms)* Insulin (0.08 unit per 100 gms)†	7	2.9±0.39	5.6±0.68	+2.7±0.46	<0.01	—	—	—
A.C.E. (1.0 ml per 100 gms)* Insulin (0.25 unit per 100 gms)†	7	1.8±0.23	4.9±0.46	+3.1±0.46	<0.01	—	—	—
A.C.E. (1.0 ml per 100 gms)* Insulin (0.5 unit per 100 gms)†	10	2.0±0.31	5.1±0.27	+3.1±0.41	<0.01	—	—	—

* 0.5 ml A.C.E./100 gms at 0 and 1 hours.

† Injected at 3rd hour

‡ Standard Error.

RESULTS

Table 1 shows the effects of A.C.E., insulin, and A.C.E. plus insulin on the rates of urea formation during successive three hour periods. Table 2 records the blood sugar levels in these experiments. 0.5 ml. A.C.E. per 100 grams body weight was injected subcutaneously at 0 and 1 hours (16 and 17 hours after nephrectomy) and insulin at the third hour in doses of 0.5, 0.25 and 0.08 unit per 100 grams body weight. In agreement with previous observations (Engel, Pentz and Engel, 1948, Engel, Schiller and Pentz, 1949) the injection of 1.0 ml of A.C.E. in divided doses at 0 and 1 hours was followed by a significant increase in urea formation during the second 3 hour period, amounting to 0.9 ± 0.28 mg. urea nitrogen per 100 grams body weight per hour. As shown previously and confirmed in Table 4, A.C.E. does not effect urea formation during the first three hours after its administration, but its effect is fully demonstrable 3-6 hours after injection. The blood sugar showed a small, but not significant increase between the 3rd and 6th hours. Insulin in doses of 0.08 and 0.5 unit per 100 grams body weight caused small but variable and hence statistically insignificant increases in urea formation during the

three hours following injection and significantly lowered the blood sugar. In the next three hour period (6-9 hours) a highly significant increase in urea formation occurred after 0.08 unit of insulin, injected at the 3rd hour. This amounted to 2.0 ± 0.23 mg. N/100 grams/hour. It is probable that this delayed increase after insulin is related to endogenous adrenal cortical hormone production. The blood sugar had risen from 40 ± 3.0 mg. per cent to 74 ± 4.6 mg. per cent (Table 2). In three series of rats treated as before with 1.0 ml A.C.E./100 grams during the first 3 hours, injection of 0.5, 0.25 and 0.08 unit of insulin at the 3rd hour was followed by a very striking increase in the rate of urea formation in all cases during the 3-6 hour period. This increase was 3-4 times as great as that after A.C.E. alone. With 0.08

TABLE 2. MEAN BLOOD SUGAR LEVELS WITH STANDARD ERRORS AFTER A.C.E. AND INSULIN IN NEPHRECTOMIZED RATS

Procedure	Number of rats	Blood sugar in Mg %		
		3rd hour	6th hour	9th hour
A.C.E. (1.0 ml per 100 gms)*	10	125 ± 2.7	144 ± 8.4	—
Insulin (0.08 unit per 100 gms)†	13	106 ± 3.3	40 ± 3.0	74 ± 4.6
Insulin (0.5 unit per 100 gms)†	11	126 ± 4.6	41 ± 8.8	—
A.C.E. (1.0 ml per 100 gms)*†				
Insulin (0.08 unit per 100 gms)†	7	97 ± 2.9	40 ± 9.2	—
A.C.E. (1.0 ml per 100 gms)*†				
Insulin (0.25 unit per 100 gms)†	7	101 ± 9.1	37 ± 6.4	—
A.C.E. (1.0 ml per 100 gms)*†				
Insulin (0.5 unit per 100 gms)†	10	100 ± 8.7	42 ± 4.5	—
Insulin (0.5 unit per 100 gms) +				
Glucose (500 mg per 100 gms)†	8	125 ± 6.9	153 ± 17.4	—
A.C.E. (1.0 ml per 100 gms)*†				
Insulin (0.5 unit per 100 gms) +				
Glucose (500 mg per 100 gms)†	7	105 ± 7.1	195 ± 30.6	
A.C.E. (1.0 ml per 100 gms)†	6	—	—	
A.C.E. (1.0 ml per 100 gms)††				
Insulin (0.08 unit per 100 gms)†	8	106 ± 5.2	38 ± 4.0	68 ± 5.4

* 0.5 ml A.C.E./100 gms injected at 0 and 1 hours.

† Injected at 3rd hour.

unit of insulin it was not significantly greater than that occurring during the 6-9 hour interval following the same dose of insulin alone. The pretreatment with A.C.E. did not prevent or decrease the hypoglycemia following insulin (Table 2).

The data in Table 3 indicate that the accelerated rate of urea formation is due to the hypoglycemia rather than to the insulin per se. Prevention of the hypoglycemia by the injection of large amounts of glucose (500 mg. per 100 grams body weight) simultaneously with 0.5 unit of insulin completely prevented the change in urea nitrogen formation previously noted after A.C.E. and insulin. The blood sugar levels at 6 hours were higher than at 3 hours in both series, significantly so in the A.C.E. treated rats (Table 2).

It was of interest to determine whether the accentuated urea accumulation after A.C.E. and insulin would occur if A.C.E. treat-

TABLE 3. INFLUENCE OF GLUCOSE ON THE UREA PRODUCTION OF NEPHRECTOMIZED RATS AFTER A.C.E. AND INSULIN

Procedure	No. of rats	Urea N—Mg N per 100 Gm. B. W./Hour			
		0-3 hours	3-6 hours	Change	P
Insulin (0.5 unit per 100 gms)† + Glucose (500 mg. per 100 gms)†	8	2.0 ± 0.26	1.8 ± 0.27	-0.2 ± 0.41	>0.5
A.C.E. (1.0 ml per 100 gms)* + Insulin (0.5 unit per 100 gms)† + Glucose (500 mg per 100 gms)†	7	2.4 ± 0.21	2.5 ± 0.20	+0.1 ± 0.37	>0.5

* 0.5 ml A.C.E./100 gms injected at 0 and 1 hours.

† Injected at 3rd hour.

ment were delayed until insulin was given. Table 4 illustrates the results of an experiment in which A.C.E. and insulin were both injected at the 3rd hour. It demonstrates that in the presence of insulin hypoglycemia A.C.E. injection is followed at once by a marked increase in protein catabolism which is sustained during the subsequent 6 hours. This is in contrast to the lack of response of the fasted rat to the same dose of A.C.E. during the first 3 hours after its administration and to the similar delay noted after insulin alone

TABLE 4. INFLUENCE OF SIMULTANEOUS A.C.E. AND INSULIN INJECTION ON THE UREA PRODUCTION OF NEPHRECTOMIZED RATS

Procedure	No. of rats	Urea N—Mg N per 100 Gm. B. W./Hour					
		0-3 hours	3-6 hours	Change	P	6-9 hours	Change
A.C.E. (1.0 ml per 100 gms)†	6	1.4 ± 0.26	1.5 ± 0.21	+0.1 ± 0.35	>0.5	—	—
A.C.E. (1.0 ml per 100 gms)† + Insulin (0.08 unit per 100 gms)†	8	2.2 ± 0.23	4.8 ± 0.37	+2.6 ± 0.44	<0.01	5.2 ± 0.50	+3.0 ± 0.53

† Injected at 3rd Hour.

(Table 1), and believed to be indicative of endogenous adrenal secretion. In the presence of the proper stimulus, i.e., carbohydrate depletion, an increased level of adrenal hormone is associated with a prompt change in protein metabolism. Under different conditions, i.e., fasting or glucose feeding, this same dose of hormone elicits either a small, delayed increase, or no change in protein catabolism.

DISCUSSION

The remarkable increase in urea formation in the nephrectomized rat following treatment with adrenal cortical extract prior to or during insulin hypoglycemia would seem to support the view previously expressed (Engel, Schiller, and Pentz, 1949, Bondy, Engel and Farrar, 1949) that relative depletion of carbohydrate stores and subsequent increased need for gluconeogenesis during fasting is at least one factor which determines the occurrence and magnitude of

the protein catabolic response to A.C.E. This interpretation was based on the finding that the increased urea formation noted after A.C.E. in the fasted nephrectomized rat persisted after the intravenous injection of a fat emulsion or of human serum albumin, but not if glucose or amino acids were injected. During either fasting or following fat injection tissue proteins were the only possible sources of new carbohydrate after A.C.E. treatment. Albumin injected intravenously presumably was indistinguishable from tissue protein as a new source of carbohydrate and hence an increase in urea formation occurred in the rat treated with A.C.E. but not in the untreated animal. After glucose no increase in urea formation occurred in the fasted or albumin injected rat pretreated with A.C.E. No greater amount of urea was formed after the injection of amino acids and A.C.E. than after either one alone and evidence was presented to indicate that the amino acid injection suppressed the tissue protein breakdown customarily seen after A.C.E. All data indicated that the effects of A.C.E., however mediated, were on protein breakdown itself and not on deamination of amino acids, an interpretation supported by the findings in adrenalectomized rats (Bondy, Engel, and Farrar 1949). In order to permit survival after combined adrenalectomy and nephrectomy, a low potassium diet was necessary. Adrenalectomized-nephrectomized rats showed the same rate of urea formation after amino acid injection as did rats with intact adrenals. Under the dietary conditions used here, the nephrectomized rat exhibited an increase in urea formation after the injection of whole protein (rat plasma), a response not obtained in rats on an adequate diet, as noted above. This was attributed, in part, to a disability in storing protein during potassium deficiency. In contrast, the adrenalectomized rat showed no change in urea formation after intravenous protein injection unless A.C.E. were given, indicating that the latter was necessary for the protein catabolism that occurred.

Insulin hypoglycemia in the fasted animal throws a severe strain on the gluconeogenetic mechanisms and hence it was anticipated that if the protein catabolic response to A.C.E. were conditioned by the needs for gluconeogenesis and the non-availability of carbohydrate precursors other than tissue protein, a large increase in urea formation would occur after A.C.E. and insulin in the nephrectomized rat. Such an increase did occur and in contrast to the effect of A.C.E. in the fasted animal, there was no latent period between the time of injection of A.C.E. and the first detectable increase in urea. Either the relative need for gluconeogenesis determines both the speed and the magnitude of the protein catabolic response to A.C.E. or the magnitude of the response to A.C.E. after fasting is so much smaller during the first three hours as to be undetectable by the present methods. The delayed occurrence of an increased rate of urea formation in the rats treated with insulin alone is of considerable interest and suggests that approximately 3 hours must elapse before endog-

eous adrenal cortical hormone secretion has reached levels sufficient to trip off the protein catabolic response. It is well known that insulin hypoglycemia in the fasted animal will increase protein catabolism and stimulate the adrenal cortex (See Tepperman, Engel, and Long, 1943 for review of literature). The present method of study has made it possible to determine the time relations of this response.

As might be anticipated from earlier work, prevention of hypoglycemia by administering glucose simultaneously with the insulin abolished the accelerated protein catabolism after A.C.E. and insulin, as well as that to be expected from A.C.E. alone. The change in urea

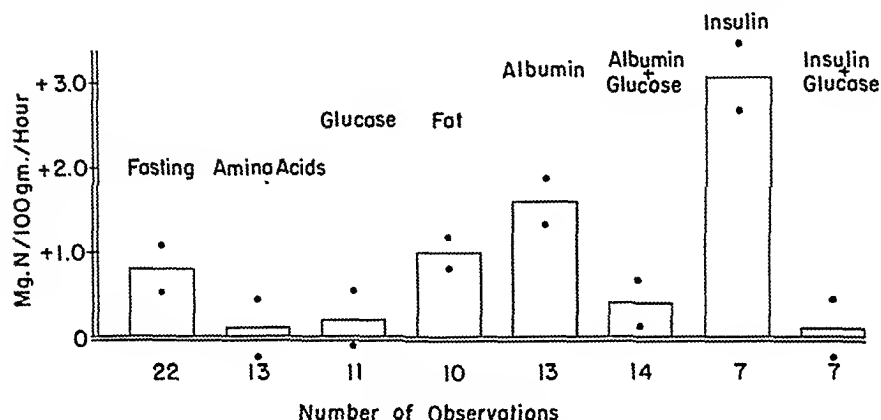


FIG. 1. Mean increases (\pm S.E.) in urea formation after treatment with 1.0 cc. of A.C.E. The vertical bars represent the differences in urea formation between animals treated with A.C.E. and controls similarly treated but for A.C.E. The data for the first six bars were taken from a previous report (Engel, Schiller and Pentz, 1949).

formation, therefore, cannot be attributed to the insulin per se. In point of fact, insulin in the presence of sufficient glucose to prevent hypoglycemia has an inhibitory effect on protein catabolism. Frame and Russell (1946) and Ingle, Prestrud and Nezamis (1947) showed that insulin will depress the rate of accumulation of amino acids in the blood of eviscerated rats. The evidence all favors the view that the increase in urea formation in the nephrectomized rat after A.C.E., with or without insulin, reflects an increase in protein catabolism that is mediated directly or indirectly by the cortical hormones. It is of interest in this regard that Bondy (1949) has recently shown that A.C.E. will increase the rate of amino acid accumulation in the eviscerated rat and Ingle, Prestrud and Nezamis (1948) report that adrenalectomy in the eviscerated rat lowered the rate of accumulation of amino acids. It is important to know whether glucose will influence amino acid levels in eviscerated rats after A.C.E. treatment.

The observations reported here and in the previous paper (Engel, Schiller and Pentz 1949) throw additional light on the mechanisms involved in the changes in protein metabolism after treatment with

adrenal cortical extract. Figure 1 records the mean increases (\pm S.E.) in urea formation after the injection of 1.0 cc. of A.C.E. into animals differently treated, and emphasizes the most significant feature of these observations. That is that the injection of the same dose of A.C.E. may be followed by widely different responses depending on the internal metabolic environment of the organism at the time of treatment. Thus, there may be no detectable change in protein catabolism, as reflected in urea formation, in the A.C.E. treated animal given amino acids, glucose, albumin plus glucose or insulin plus glucose, a moderate increase in the fasted, the fat or albumin treated animal and a marked increase during insulin hypoglycemia. These facts suggest that the adrenal cortex may not be the prime mover in changing the rate of protein catabolism. Rather it would appear that a definite level of cortical hormone may be necessary but not responsible for increasing the rate of protein catabolism in response to the proper stimulus but that the magnitude of the response is largely independent of the amount of cortical hormone beyond the stimulating level provided overdosage effects are not involved. This implies that the effects of massive doses of cortical hormone may not be the same as those after physiologic doses. There is ample precedent for such a view. Thyroid is growth promoting in physiological doses, growth inhibiting in excess. Similarly, insulin has different effects at different dose levels. The concept that the adrenal cortex is necessary but not responsible for a change in protein metabolism is the same as that expressed by Ingle, Ward and Kuizenga (1947) with reference to the relation of the adrenal cortex to the catabolic response to injury. They showed that the increase in nitrogen excretion which occurs in normal but not in adrenalectomized animals after a fracture, did occur in the latter if given a constant maintenance dose of A.C.E. Indeed, one may well wonder whether stress in its broadest sense, which would include fasting as well as insulin hypoglycemia in our experiments, may not be one common denominator determining whether an increase in protein catabolism occurs in the presence of cortical hormone. The magnitude of the stress and the availability of new carbohydrate from precursors other than tissue protein might largely determine the magnitude of the protein catabolic response. Since all types of stress are followed by evidence of increased protein catabolism and activation of the adrenal cortex in normal but not in adrenalectomized or hypophysectomized animals (Tepperman, Engel and Long, 1943), whereas stress regularly results in hypoglycemia and death in the latter, it may be that an increased need for carbohydrate is the first effect of stress in general. In the presence of a normally responsive adrenal cortex the counteraction of increased protein catabolism, gluconeogenesis, and decreased carbohydrate utilization would then take place as existing stores of carbohydrate begin to become depleted. It should be emphasized that these interpretations of the role of the adrenal cortex in metabolism

refer to protein metabolism alone. In this regard, however, it is of interest that Levin (1949) has recently reported that the increase in liver fat, which begins within an hour of physical stress or injection of adrenocorticotrophic hormone, and which he believes is mediated through the pituitary-adrenal system, is prevented by parenteral administration of glucose during the stress or after A.C.T.H.

Further investigation is in progress with respect to the role of stress in the protein catabolic response to A.C.E.

SUMMARY

The effects were studied of insulin and adrenal cortical extract (A.C.E.) on protein catabolism, measured as the rate of urea accumulation in nephrectomized rats.

Both A.C.E. and hypoglycemic doses of insulin each caused a delayed increase in urea formation beginning 3 hours after injection. The response to insulin was significantly greater than that to A.C.E. and was interpreted as being related to endogenous cortin production.

Either pretreatment with A.C.E. followed in 3 hours by insulin or simultaneous injection of A.C.E. and insulin resulted in a marked increase in urea formation beginning immediately after injection of insulin. The increase after A.C.E. plus insulin was 3-4 times as great as after A.C.E. alone, but not significantly greater than the delayed response to insulin.

Glucose prevented both the hypoglycemia and the increase in urea formation seen after insulin and A.C.E.

Since hypoglycemia greatly accentuates the needs for new carbohydrate, the finding of greatly accelerated protein catabolism after insulin and A.C.E., compared to A.C.E. alone, supports the view that the need for new carbohydrate is one factor which determines the occurrence and magnitude of the protein catabolic response to A.C.E. The possible role of nonspecific stress in this response is considered and the possibility suggested that this may be the factor which determines the protein catabolic response for which the adrenal cortex is necessary but not itself responsible.

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TROPHIC HORMONES OF THE PLACENTA: FAILURE TO DEMONSTRATE THYROTROPHIN OR ADRENOCORTICOTROPHIN PRODUCTION IN THE HYPOPHYSECTOMIZED PREGNANT RAT¹

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IT HAS BEEN KNOWN since the work of such investigators as Allan and Wiles (1932) using the cat and Pencharz and Long (1933) using the rat that pregnancy can continue in gravid animals after hypophysectomy. This observation has been confirmed in many different species such as guinea pigs, mice, dogs, rabbits, and monkeys. It is now well established, of course, that pregnancy is able to continue because the placenta takes over in part the functions of the pituitary gland and ovary. In the lower mammals investigated, the placental hormone chiefly responsible for maintaining pregnancy seems to be a luteotrophin, since ovariectomy in animals such as the rat results in abortion or resorption of the fetuses in hypophysectomized pregnant animals although pregnancy continues undisturbed if the ovaries are left intact. In certain species including the primates, the placenta is able also to take over the function of the ovaries, since ovariectomy will not terminate pregnancy and estrogen and progesterone have been isolated from placental tissue of the higher mammals.

It is a curious fact that the pregnant animals deprived of their pituitaries seem to do remarkably well in comparison to the notably poor behavior of the hypophysectomized non-pregnant animals. For instance, Gardner and Allen (1942) found that mice hypophysectomized on the tenth day after mating maintained parallel increases in body weight with their non-operated pregnant controls. It is almost as if the pregnant animal were able to maintain a normal endocrine balance in the absence of the pituitary. Except for the discoveries that the placenta secretes a luteotrophin (Astwood and Greep, 1938), estrogen (Doisy *et al.*, 1924), progesterone (Ehrhardt, 1934), and androgen (Cunningham and Kühn, 1941), little work has been done on the problem of other endocrine functions of the placenta.

Because the pregnant rat seems to thrive in the absence of the pituitary and since the rat placenta is apparently unable to substitute

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for the ovaries by secreting sufficient amounts of estrogen and progesterone to maintain pregnancy, the present investigation was undertaken to determine whether the pregnant rat is able to maintain endocrine glands other than the corpus luteum in a normal functional state. Since quite satisfactory methods are now available for evaluating thyroid and adrenal function, this work was limited to a study of whether or not a thyrotrophin or an adrenocorticotrophin are secreted in the hypophysectomized pregnant rat.

TABLE 1. THYROID/SERUM (T/S) IODIDE RATIO AND THE WEIGHTS OF CERTAIN ENDOCRINE GLANDS IN MG./100 GM. BODY WEIGHT

	Number of animals	T/S	Adrenal	Ovary	Thymus	Thyroid
I Pregnant, intact	9	219.6 ± 29.9	22.8 ± 1.4	40.2 ± 1.6	59.9 ± 5.5	14.1 ± 1.1
II Pregnant, hypophysectomized	9	6.4 ± 0.7	18.6 ± 0.6	37.8 ± 1.8	95.3 ± 16.3	6.6 ± 0.5
III Non-pregnant, hypophysectomized	10	6.1 ± 1.1	20.4 ± 1.6	21.6 ± 0.8	139.3 ± 13.7	7.0 ± 0.6

MATERIALS AND METHODS

Female, 150–200 gm., Sprague-Dawley rats of the Harvard strain were used in all experiments. Thirty-five rats were used in all, but some had to be discarded because of incomplete hypophysectomies. The rats were divided into three groups: Group I consisted of nine unoperated pregnant rats, Group II contained nine pregnant rats which were hypophysectomized by the parapharyngeal approach on the twelfth to thirteenth day after mating, Group III comprised ten non-pregnant hypophysectomized animals. All pregnant rats were killed by ether anesthesia on the twenty-first day of pregnancy. The non-pregnant animals were killed in the same manner on the eighth to ninth day following hypophysectomy. The hypophysectomized animals were given 0.03 per cent propylthiouracil mixed in mink chow immediately following the operation. The intact, pregnant animals were placed on the propylthiouracil diet on the twelfth to thirteenth day of pregnancy. Prior to the administration of the antithyroid drug the animals were fed on Purina fox checkers. Body weights of the pregnant animals were determined after removal of the fetuses and placentae. The thyroids, adrenals, ovaries, and thymus were removed, dissected free of fat and connective tissue on moist blotting paper and immediately weighed to the nearest 0.1 mg. on a Roller-Smith torsion balance, following which all except the left lobe of the thyroid were deposited in 10 per cent formalin.

The thyroid/serum iodide ratio was determined in the following manner. Each animal was given a subcutaneous injection of 5 mg. propylthiouracil in an alkaline, aqueous solution on the last day of the experiment. Thirty to sixty minutes later a tracer dose of 2 to 3 microcuries of carrier-free I^{131} was injected subcutaneously. One hour later the animals were killed with ether anesthesia as described above. Heart blood was obtained, either by cardiac puncture or by opening the thorax and incising the heart directly, and this was centrifuged to obtain the serum. 0.1 cc. of this serum was pipetted onto

a piece of absorbent paper not quite covering a one-inch standard copper disc. The serum was evaporated to dryness under an infra-red lamp and the paper then secured in place by covering with a single layer of Scotch Tape.

The left lobe of the thyroid gland was placed on a similar piece of absorbent paper on a copper disc immediately after weighing. It was then covered with a single layer of Scotch Tape and the gland smashed flat by pressing with a heavy weight. The radioactivity of each sample was determined with a mica-windowed, Geiger-Müller counter. The radioactivity per 100 mg. of each sample was then computed, and the thyroid/serum (T/S) iodide ratio thereby determined.

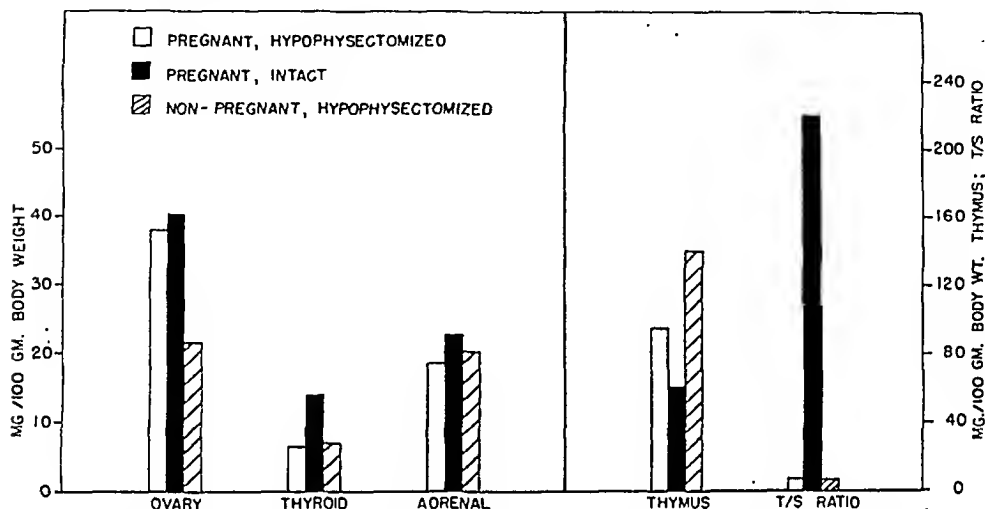


FIG. 1. Graphic comparison of certain organ weights and the thyroid/serum iodide ratio of the three groups of experimental animals.

After fixation in formalin for at least one week, the adrenal glands were washed in running water for an hour and sectioned on the freezing microtome at 15 micra.² One of the sections was stained with sudan IV, one with sudan black B, and a third treated with the Schiff plasmal reagent. An unstained section and a control section extracted for one-half hour in cold acetone were examined in the polarizing and fluorescent microscopes to determine the quantity and distribution of acetone-soluble, birefringent, and auto-fluorescent materials in the cortex. The lipids which react in a positive manner to all of these tests are generally conceded to give a fair indication of the hormone present in the gland.

After the animals had been killed, the cranium was opened and the region of the sella turcica examined carefully under a dissecting microscope in order to determine the completeness of hypophysectomy. All operated animals in which there was any question of a pituitary fragment remaining were excluded.

RESULTS

As had been found by previous investigators, the hypophysectomized pregnant animals experienced no difficulty in carrying their

² The adrenal sections were kindly prepared through the courtesy of Dr. R. O. Greep.



FIG. 2. Sudan black B stain of frozen section of adrenal from intact pregnant rat. The glomerulosa and fasciculata are directly contiguous without any intervening unstained cells.

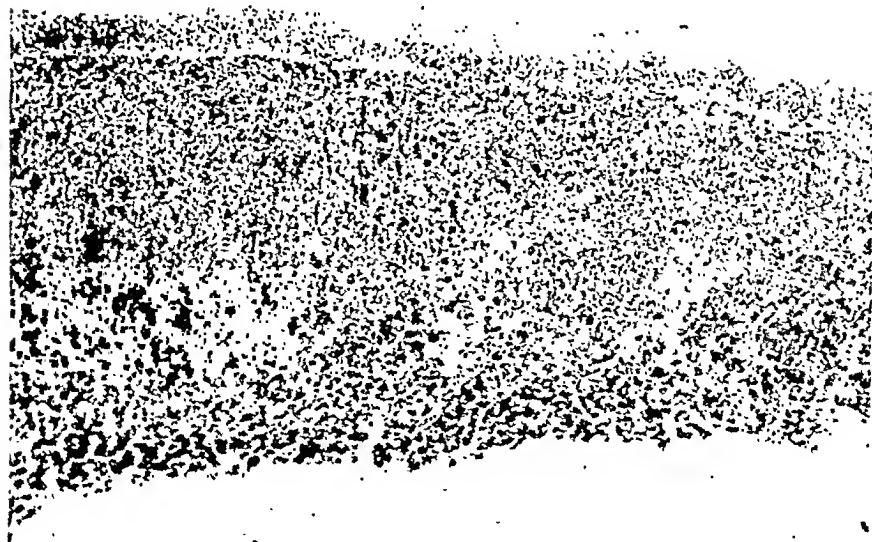


FIG. 3. Sudan black B stain of adrenal from hypophysectomized pregnant rat. Note the appearance of a "lipophobic" zone between the glomerulosa and fasciculata.

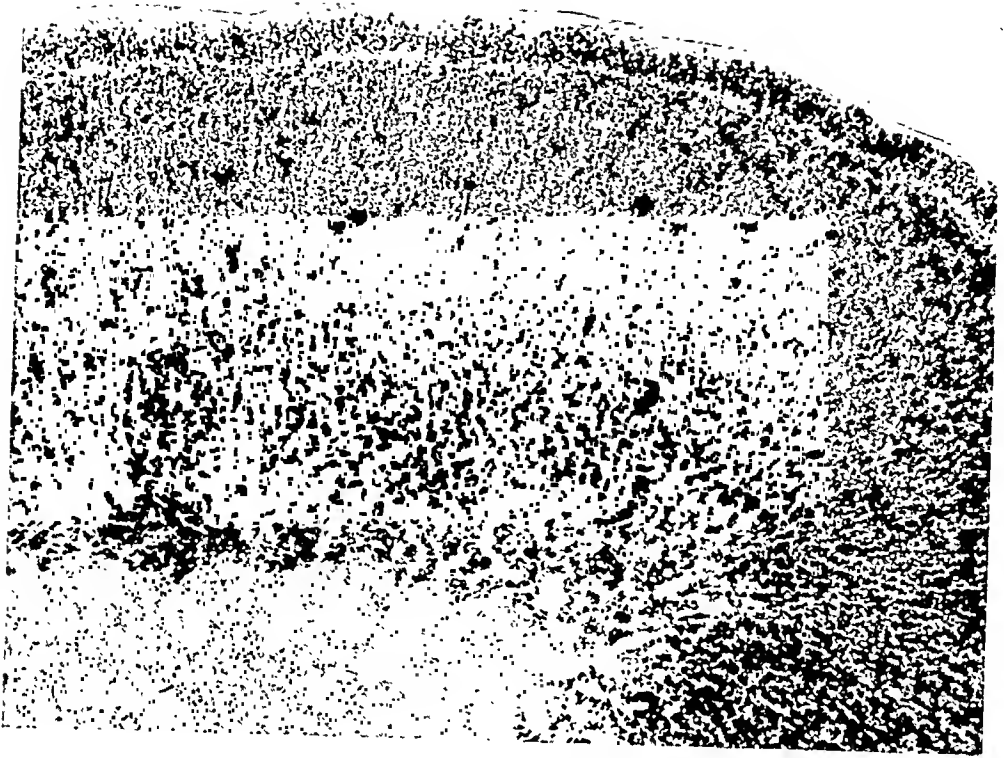


FIG. 4. Sudan black B stain of adrenal from hypophysectomized non-pregnant rat. The appearance is identical to that in Figure 3.

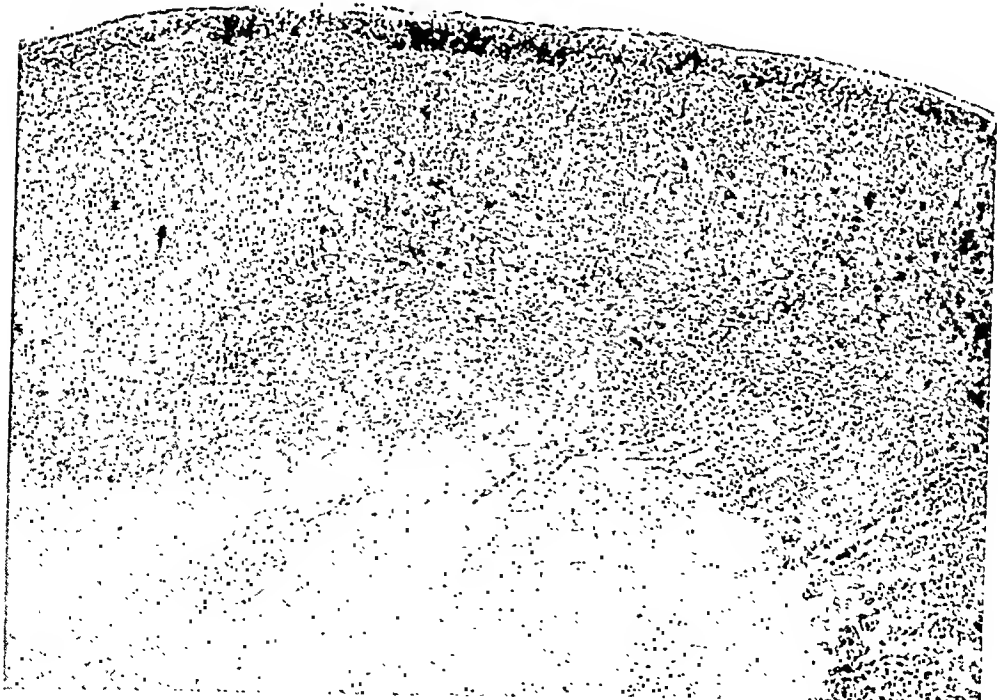


FIG. 5. Schiff stain of adrenal from intact pregnant rat. Note that the glomerulosa is more intensely stained than the fasciculata.

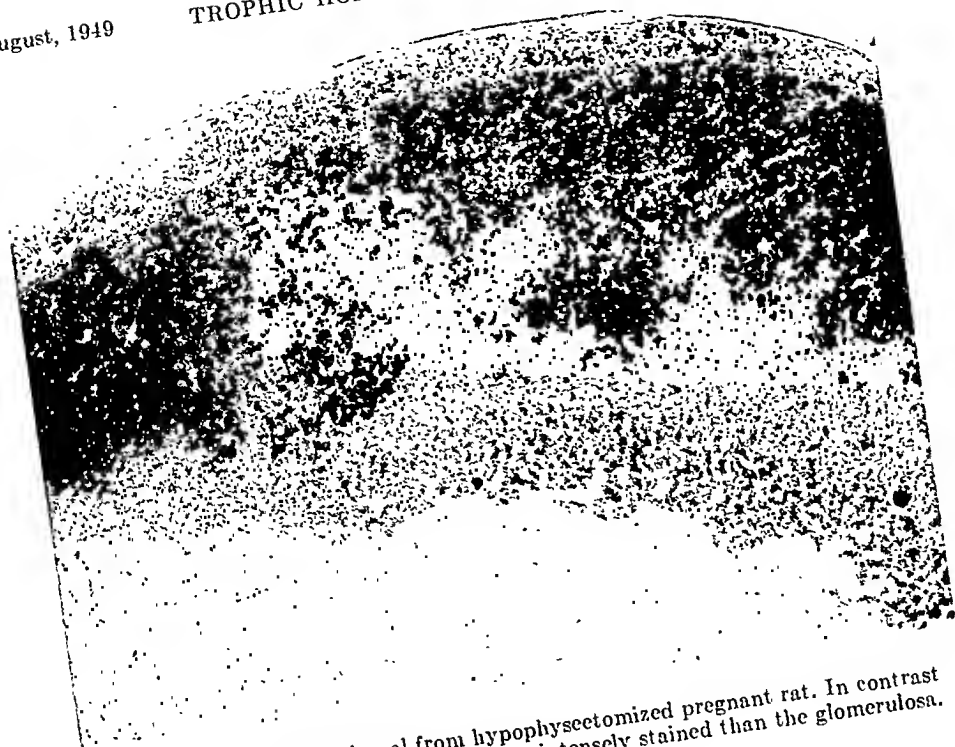


FIG. 6. Schiff stain of adrenal from hypophysectomized pregnant rat. In contrast to Figure 5, the fasciculata is much more intensely stained than the glomerulosa.

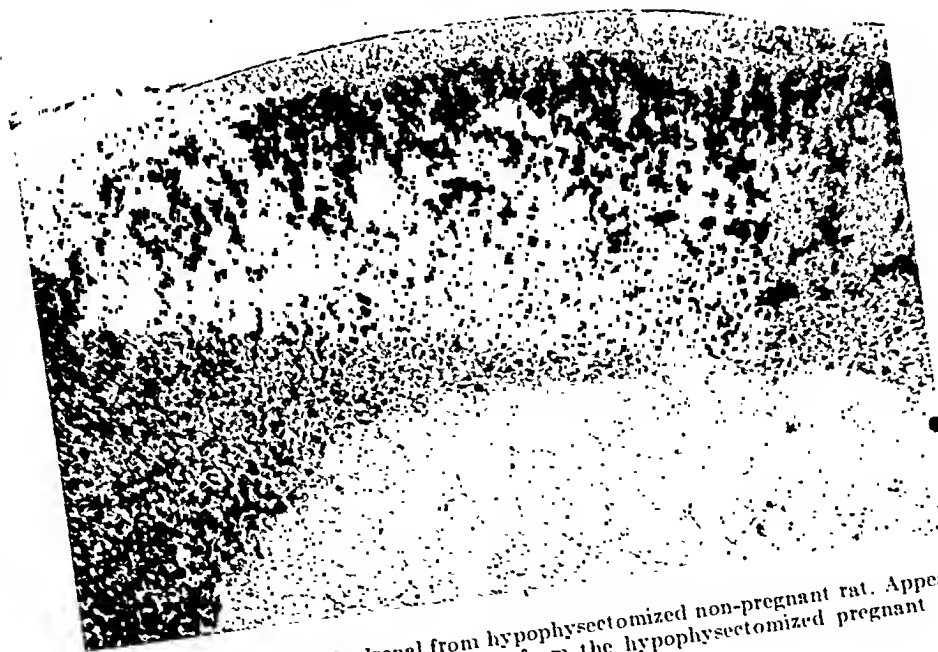


FIG. 7. Schiff stain of adrenal from hypophysectomized non-pregnant rat. Appearance is identical to that of the adrenal from the hypophysectomized pregnant rat illustrated in Figure 6.

foetuses to term. Since Pencharz and Long (1933) had shown that hypophysectomy prior to the eleventh day of pregnancy usually resulted in resorption or expulsion of the foetuses, the twelfth to thirteenth day after mating was chosen for the operation in these experiments. In no instance was any resorption or maceration of any of the young noted at autopsy, nor was the number of animals per litter reduced below the figure for the controls. The body weights of the hypophysectomized pregnant animals were comparable to those of the intact animals, while the hypophysectomized non-pregnant animals seemed to be somewhat more emaciated.

Thyrotrophin

VanderLaan and VanderLaan (1947) have shown that the normal thyroid/serum iodide ratio of 25:1 could be increased ten-fold by placing rats on a diet of 0.03 per cent propylthiouracil for ten days or longer. Recently, VanderLaan and Greer (to be published) have shown that this marked increase in the iodide-concentrating power of the thyroid was due to the increased efficiency of the thyroid cells when stimulated by the large amounts of thyrotrophin secreted by the pituitary of a hypothyroid rat. Hypophysectomy would reduce the T/S ratio to the normal level within two days in rats continued on propylthiouracil. The iodide-concentrating mechanism was independent of the cellularity of the gland and of its relative mass. Thyrotrophin injection would cause an increase in the T/S ratio similar to that produced by chronic propylthiouracil administration.

The determination of the thyroid/serum iodide ratio therefore seemed an excellent method for estimating just how much thyrotrophin was being produced in these animals. It was reasoned that the feeding of propylthiouracil from the time of hypophysectomy until the time of autopsy would serve to accentuate any differences between the groups which might exist. If, as seemed unlikely, the placenta were capable of secreting as much thyrotrophin in response to the stimulus of thyroxin-lack as was the pituitary, the hypophysectomized pregnant animals would exhibit T/S ratios comparable to the markedly elevated levels in the intact hypothyroid animals. On the other hand, if the placenta were able to secrete only enough thyrotrophin to maintain a euthyroid state under normal conditions a T/S ratio of around 25:1, approximating that of untreated intact animals, would be expected. If no thyrotrophin were secreted by a pregnant rat in the absence of the pituitary, the T/S ratio should fall well below 25:1.

a) *Pregnant, intact animals*: The increase in the T/S ratio observed in these animals was no different from that previously observed in non-pregnant, thiouracil-treated rats. The mean value was 219.6:1 (range 89.3-382).

b) *Pregnant, hypophysectomized animals*: These animals gave no

indication of any extra-pituitary source of thyrotrophin. The mean ratio was 6.4:1 (range 3.3–13.6) which represents a level only one-fourth that of intact animals which were not treated with thiouracil.

c) *Non-pregnant, hypophysectomized animals*: As expected, no evidence of thyrotrophin production in these animals was found. The mean T/S level was 6.1:1 (range 1.8–10.1) almost identical to that in b), above.

Adrenocorticotrophin

Unfortunately, no reliable and simple method has yet been found for directly measuring the adrenocorticotrophin production in small animals. Histochemical methods, however, are generally conceded to give a fairly reliable index of adrenal cortex activity. Adrenal weights have been considered to be of great value in estimating the amount of adrenocorticotrophin present, but in these experiments there was very little difference between the weights of the adrenals in any of the three groups. This may well have been because all animals were receiving antithyroid medication for it has been well established (Deane and Greep, 1947) that thiouracil will cause a decrease in adrenal size.

In any event, examination of the adrenal sections showed that although the intact pregnant rats had perfectly normal appearing glands, both the pregnant and non-pregnant hypophysectomized animals showed changes in the cortex characteristic of rats operated on in this manner. There was a narrowing of the fasciculata, and the lipid droplets in this region were much more diffuse and less localized in intensely staining cells than in the intact animals. A characteristic lipophobic zone appeared between the glomerulosa and fasciculata of the hypophysectomized animals which was not seen in the intact animals. The Schiff reagent stain revealed a darker staining glomerulosa and lighter staining fasciculata for the intact animals and just the reverse for both groups of operated animals. It was concluded, therefore, that no adrenocorticotrophin could be detected in the hypophysectomized pregnant rat.

Weights of endocrine glands

Although they were not used as such in this investigation, the weights of organs which depend upon the pituitary for their function have frequently been employed as an index of pituitary activity. The findings in these experiments also show that in the absence of trophic hormones the "end-organs" tend to atrophy. For instance, the thyroid and adrenal weights in the hypophysectomized animals are below those of the intact rats, although in the case of the adrenals it is questionable whether a significant difference exists. The thymus, which has been observed to enlarge after adrenalectomy or hypophysectomy, was also a good deal larger in the hypophysectomized than

the intact rats in these experiments. The ovaries, on the other hand, which were stimulated by the placental gonadotrophins, were markedly larger in both groups of pregnant rats than in the non-pregnant. The large size of the thyroids of the intact pregnant animals, of course, was due to the administration of propylthiouracil.

DISCUSSION

These results strongly indicate that neither thyrotrophin nor adrenocorticotrophin is produced in the hypophysectomized pregnant rat. In the only other investigation along a similar line which could be found in the literature, Gardner and Allen (1942) observed that the adrenals of pregnant mice which were hypophysectomized on the tenth day after mating and killed on the first to second day after parturition had adrenals weighing only 3.8 mg. compared to 6.9 mg. for the pregnant, intact controls. The loss of adrenal substance in their experiment was also chiefly in the fasciculata. The fact that they observed more adrenal atrophy in their work than occurred in the present investigation may have been due to any or all of three factors: 1) a species difference may exist between the rate of atrophy of the adrenals of mice and rats; 2) their animals were allowed to go two to four days longer after hypophysectomy than those in the present experiment; 3) our rats received propylthiouracil beginning at the time of operation which may have caused a reduction in adrenal size of the intact animals that would lessen any apparent loss of substance due to hypophysectomy (Deane and Greep, 1947).

If adrenocorticotrophin and thyrotrophin are not secreted by the placenta, the difficulty still arises in trying to explain why these pregnant hypophysectomized animals are able to maintain themselves so well. It is possible that although trophic hormones other than gonadotrophins are not secreted by the placenta, other hormones, such as adrenal steroids, etc., might be and would thus explain the maintenance of parallel weight gains of the hypophysectomized with the intact pregnant animals. Since steroids such as progesterone, estrogen, and androgen have been found in placental tissue, it does not seem too far fetched to postulate that other steroids as well as certain non-steroid hormones similar to gonadotrophins may yet be discovered in this organ. That the good health of the hypophysectomized pregnant rats may have something to do with the placenta other than its luteotrophic function is further indicated by the work of Cutuly (1942) who found that in hypophysectomized rats in which pregnancy was maintained by injections of pituitary synergist or lactogenic hormone the animals gained in weight as did their unoperated, pregnant controls. After delivery or after the foetuses had died in utero, however, there was a precipitate decrease in body weight.

SUMMARY

Pregnant rats were hypophysectomized on the twelfth to thirteenth day after mating and given a 0.03 per cent propylthiouracil diet. They were killed on the twenty-first day of pregnancy. The level of thyroid function was determined by the thyroid/serum iodide ratio and of adrenal function by histochemical studies. No evidence of thyrotrophic or adrenocorticotrophic activity in the animals thus treated was found.

ACKNOWLEDGMENTS

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THE EXCRETION OF PANTOTHENIC ACID AND ASCORBIC ACID BY INTACT AND ADRENALECTOMIZED RATS ON DIETS SUPPLEMENTED WITH AND DEFICIENT IN PANTOTHENIC ACID

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INTRODUCTION

PROLONGED survival of adrenalectomized rats receiving 1% NaCl as drinking water and a diet supplemented with at least 4 mg. of calcium pantothenate daily has previously been reported (Ralli, 1946; Dumm and Ralli, 1948). These rats appeared well, gained weight, and were capable of muscular work. In view of the findings, studies are being conducted in adrenalectomized rats to establish the role that pantothenic acid plays under these circumstances. The present report is concerned with the excretion of pantothenate and of ascorbic acid by adrenalectomized and intact rats on diets deficient in and supplemented with calcium pantothenate.

EXPERIMENTAL

All rats were bred in the laboratory. To deplete the rats of pantothenic acid they were placed on a diet deficient in pantothenic acid (Ralli and Graef, 1943; Dumm and Ralli, 1948) at 30 days of age and were continued on this diet for 30 days. The excretion of pantothenate was determined at intervals during this period. At 60 days of age a group of the animals were adrenalectomized and these, as well as the remaining intact rats, were then placed on a diet supplemented with 4 mg. of pantothenic acid daily and 1% NaCl as drinking water. The excretion of pantothenate was determined in both groups of rats at intervals for 115 days. Following this period 4 adrenalectomized and 2 intact rats were returned to the pantothenic acid deficient diet and the excretion of pantothenate was followed for another 30 days.

The excretion of ascorbic acid was determined in all groups of rats under the same conditions of diet and adrenalectomy. In addition ascorbic acid excretion was determined in a group of normal male and female rats at different ages while on the Nuchow diet. This was done in order to establish the effect of sex and age on ascorbic acid excretion.

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All 24-hour urines were collected from single rats in metabolism cages. Specimens to be assayed for pantothenate were preserved with toluene. Urines in which ascorbic acid was to be determined were collected with 1 cc. of 5 N H_2SO_4 (containing .01 M hydroxyquinoline) for each 10 cc. of urine expected.

The pantothenate determinations were carried out by microbiological assay methods (Atkin *et al.*, 1944; Skeggs and Wright, 1944) in the Nutrition Laboratories of Hoffmann-LaRoche, Inc., under the direction of Dr. Saul H. Rubin. Ascorbic acid was determined in the urine by the method described by Rowe and Kuether (1943).

TABLE 1. THE EXCRETION OF PANTOTHENIC ACID BY ADRENALECTOMIZED AND INTACT RATS

Pantothenic Acid Intake 4.0 Mg./Day Following a 30-day Deficient Period

Intact Rats				Adrenalectomized Rats		
Age days	Days on Pan. ac.	Pan. ac. excreted mg.	% Pan. acid excreted	Days after adr. & on pan. acid	Pan. acid excreted mg.	% Pan. acid excreted
63	5	2.5	61	5	3.3	82
64	6	3.0	75	6	4.0	100
65	7	3.5	89	7	1.6	41
66	8	2.4	59	8	2.9	73
72	14	1.8	46	14	1.9	48
72	14	2.0	48	14	2.8	71
79	21	1.6	41	21	1.9	48
79	21	2.3	57	21	2.4	60
175*	115	1.9	41	115	2.3	62

Mean \pm S.D. 60 \pm 15. 65 \pm 20

Excretion of pantothenic acid after 20-30 days on the deficient diet was 0.002 to 0.005 mg. per rat per day.

* Rats had been receiving 3 mg. pantothenic acid daily at this time.

RESULTS

Pantothenate Excretion

After 20 to 30 days on the diet deficient in pantothenic acid the excretion of pantothenate varied from 0.002 to 0.005 mg. per day per rat. The data on the excretion of pantothenate by the intact and the adrenalectomized rats receiving 4 mg. of pantothenic acid daily are summarized in Table 1. Both groups of rats excreted about 60% of the pantothenate intake and there was no significant difference in excretion between the intact and adrenalectomized rats. The excretion of pantothenate in the adrenalectomized and intact rats following withdrawal of pantothenic acid from the rat at 119 days is shown in Figure 1. The logarithm of the pantothenate excreted in mcgm. per day is plotted against the time in days during which the diet was deficient in pantothenate. Ten days after stopping the vitamin the excretion had decreased to 0.005 to 0.013 mg. daily. It is clear that during this second period of deficiency the excretion of pantothenate by

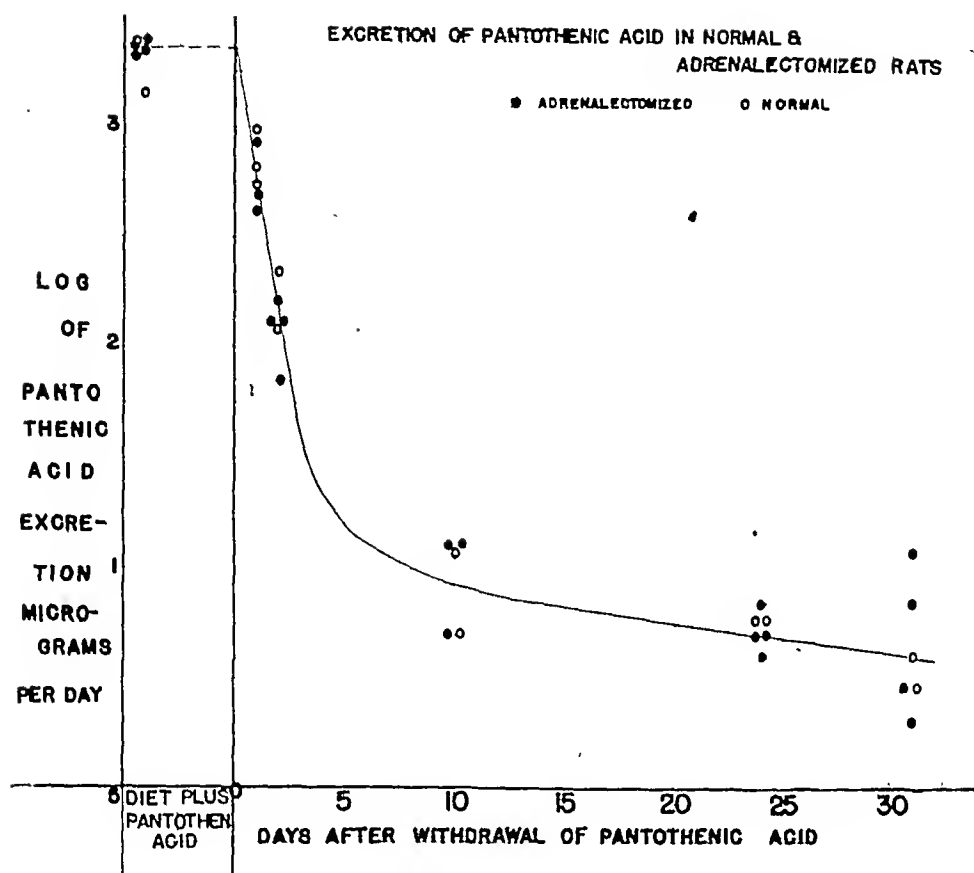


FIG. 1. Excretion of pantothenate by normal and adrenalectomized rats. The logarithm of the pantothenate excreted in micrograms per 24 hours is plotted against the time in days during which the diet was deficient in pantothenate. The rats had been maintained on a diet supplemented with 3 mg. of pantothenate daily for 119 days before pantothenate was withdrawn from the diet.

both the intact and adrenalectomized rats was essentially the same. At the conclusion of this experiment the completeness of adrenalectomy was checked by withdrawing NaCl. All adrenalectomized rats died within 3 days of NaCl withdrawal.

Ascorbic Acid Excretion

In Figure 2 data are presented on the excretion of ascorbic acid by normal rats from 30 to 103 days of age on the Nuchow diet. There were 12 rats in the group studied, 6 males and 6 females. Each point represents an average of 3 to 12 determinations. The ascorbic acid excreted is expressed as mg. per 24 hours per 100 gm. of body weight of the rat and is plotted against the age of the rat in days. All values were corrected for the small amount (7 mcgm/gm.) of ascorbic acid present in the Nuchow diet.

The excretion of ascorbic acid at 30 days of age was about 2 mg. daily and this increased to a maximum of 3.01 ± 1.40 for males and 4.08 ± 0.60 for females at about 45 days of age. Thereafter the ascorbic

acid output decreased rapidly for about 20 days (1.86 ± 0.84 for males; 2.15 ± 0.58 for females at 63 days) and then continued to fall more slowly for the duration of the study. The excretion of ascorbic acid between 40 and 50 days of age was greater by the female than by the male rats but the difference between the sexes may not be significant ($t=1.875$; $p = <0.1, >0.05$). After 65 days the average output of ascorbic acid by the male and female rats was approximately the same (1.40 ± 0.57 for males; 1.30 ± 0.93 for females at 92 days) when expressed with reference to the body weight of the animals.

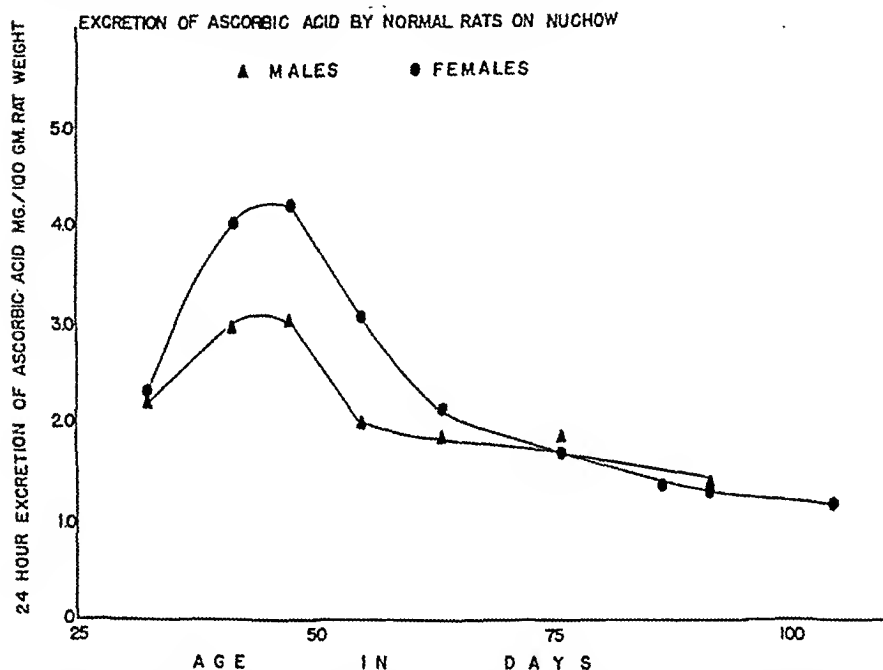


FIG. 2. Excretion of ascorbic acid by normal rats on Nuchow. The excretion of ascorbic acid in milligrams per 100 grams of rat weight per 24 hours is plotted against the age of the rat in days.

The excretion of ascorbic acid by the adrenalectomized rats and their intact controls on the experimental diet supplemented with calcium pantothenate daily is shown in Figure 3. Each point represents an average of 4 to 14 determinations at the indicated ages. It should be noted that the ordinate units in Figure 3 represent half as much ascorbic acid excreted as the ordinate units in Figure 2. The data indicate that the adrenalectomized rats excreted less ascorbic acid at all ages examined than the intact rats on the same diet. At 170 days, for example, the intact rats excreted 0.69 ± 0.25 mg. and the adrenalectomized rats 0.27 ± 0.09 mg. of ascorbic acid daily. The intact rats showed a maximum excretion of ascorbic acid between 80 and 90 days of age (1.41 ± 0.36 mg.). The adrenalectomized rats also excreted slightly more ascorbic acid between 80 and 90 days of age than during

any other time studied, but the difference between 85 days (0.53 ± 0.12 mg.) and either 75 (0.25 ± 0.10 mg.) or 95 (0.28 ± 0.18 mg.) days is probably not significant ($t = 1.556$; $p = < .2, > .1$).

Table 2 shows the excretion of ascorbic acid during part of the pantothenate withdrawal experiment described in connection with Figure 1. The daily excretion of ascorbic acid was determined for each rat 6 and 13 days before pantothenate was discontinued and on the

TABLE 2. EXCRETION OF ASCORBIC ACID
Mg. per 100 Gm. Body Weight per 24 Hours

Panto- thenate per day, mg.	Days after panto- thenate discontinued	Adrenalectomized				Intact	
		Rat A-1	Rat A-2	Rat A-3	Rat A-4	Rat I-1	Rat I-2
3	—	0.17	0.17	0.25	0.32	0.56	0.56
3	—	0.17	0.15	0.27	0.37	1.07	0.56
0	4	0.16	0.17	0.23	0.38	0.57	0.55
0	8	0.21	0.18	0.28	0.32	0.99	0.49

ASCORBIC ACID EXCRETION BY INTACT AND ADRENALECTOMIZED RATS ON DIETS
SUPPLEMENTED WITH 4 MG PANTOTHENIC ACID DAILY

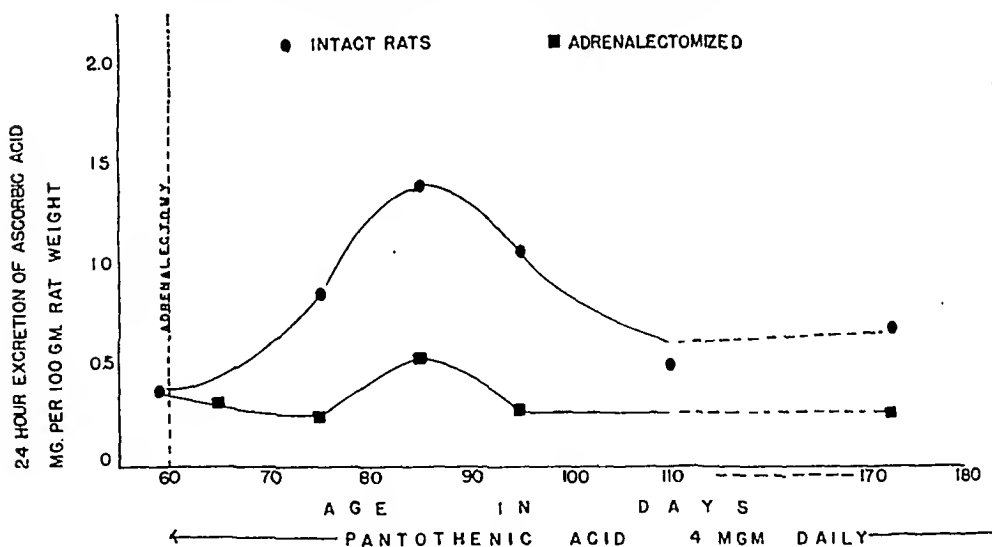


Fig. 3. The excretion of ascorbic acid by intact and adrenalectomized rats on a diet supplemented with 4 mg. pantothenate daily. The excretion of ascorbic acid in milligrams per 100 gms. of rat weight per 24 hours is plotted against the age of the rat in days. The rats received a diet deficient in pantothenate from 30 to 60 days of age and a diet supplemented with 4 mg. pantothenate daily after 60 days of age.

4th and 8th day afterward. All the adrenalectomized rats maintained a constant and low output of ascorbic acid whether or not pantothenic acid was included in the diet. More fluctuation was encountered in the ascorbic acid excretion of the intact rats, but there was no indication of any consistent change following the withdrawal of pantothenic acid in either group of animals.

DISCUSSION

In spite of the high requirement by adrenalectomized rats for pantothenic acid (Ralli, 1946; Dumm and Ralli, 1948) the data presented on its excretion by intact and by adrenalectomized rats showed no differences between the 2 groups, either at high intakes of the vitamin or in the withdrawal experiment. Both groups of rats excreted a far higher proportion of the dose of calcium pantothenate administered than has been reported in human subjects (Rubin *et al.*, 1948; Gershberg, Rubin and Ralli, 1949).

The excretion of ascorbic acid, on the other hand, was influenced both by the nature of the diet and by the removal of the adrenal glands, the latter being associated with the lowest excretion of ascorbic acid encountered. These findings are of interest for several reasons. Ascorbic acid is synthesized by the rat and is not a dietary requirement for this animal. The data presented suggest that the adrenal gland is involved in the synthesis of ascorbic acid. This is demonstrated in both of the experimental conditions to which the rats were subjected. From 30 to 60 days of age the rats received a diet deficient in pantothenic acid, a deficiency which in young rats has been shown to cause lipid depletion of the adrenal cortex (Deane and McKibben, 1946). The 24-hour excretion of ascorbic acid at the end of this period was 0.37 ± 0.18 mg. per 100 gm. of rat weight as compared to 2.00 ± 0.72 mg. by normal rats of the same age on Nuchow. When the deficient rats were adrenalectomized, the ascorbic acid continued at a very low level in spite of the addition of large amounts of pantothenate to the diet, whereas the excretion by the intact controls rose although not to the level encountered in the intact rats on Nuchow. In the intact experimental rats the excretion levelled off at about 110 days and averaged 0.9 mg. per 100 gm. of rat weight. In the adrenalectomized rats, however, the level of excretion, except for the slight increase at 85 days of age, remained at an average of 0.26 mg. per 100 gm. of rat weight and was remarkably constant, as can be seen in Table 2.

Other observers have reported that the composition of the diet can influence the excretion of ascorbic acid and have found that the cyclic compounds of the terpene and sesquiterpene series stimulated a marked increase in the amounts of ascorbic acid excreted by normal rats (Longenecker *et al.*, 1939). In our experiments the dietary variant was pantothenic acid, and although a deficiency of this substance in the young rats was associated with a marked decrease in the ascorbic acid excretion, its withdrawal from the diet of the rats, once they had reached maturity, was not associated with any striking change in excretion. This may reflect the fact that the requirement for pantothenic acid decreases with age in the rat (Unna and Richards, 1942).

There is good evidence that the age of the animals is a factor in the excretion, and probably the synthesis, of ascorbic acid. In the normal

rats the daily excretion of 3-4 mg. ascorbic acid between 40 and 50 days of age was followed by a much lower output ($< 2\text{mg.}$) after 60 days. The greatest excretion of ascorbic acid occurred just prior to the development of sexual maturity, which usually occurs between 50 and 60 days of age (Farris, 1942), suggesting that the activity of other endocrine glands is associated with the utilization and synthesis of vitamin C. This is also borne out by the work of other investigators. Plasma ascorbic acid was increased during oestrus in the cow (Phillips *et al.*, 1941) and was decreased in both the cow and the bull following the injection of gonadotropin (Erb and Andrews, 1942).

The effect of pantothenic acid on survival in adrenalectomized rats is not explained by these experiments. Apparently the decreased synthesis of ascorbic acid, as reflected by the low excretion, does not jeopardize the survival. Experiments on the acetylation of para-aminobenzoic acid are now being done and will be reported later.

SUMMARY

The data presented show that the survival of adrenalectomized rats, which occurs when they receive NaCl and large doses of pantothenic acid, is not associated with any difference in the excretion of pantothenic acid as compared to intact rats on similar diets. The excretion of ascorbic acid, however, is lower by the adrenalectomized rats receiving pantothenic acid than by their controls, but this lower excretion is apparently not a factor detrimental to prolonged survival. There was never any evidence in the animals of signs suggestive of ascorbic acid deficiency.

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THE EFFECT OF FOLLICLE STIMULATING HORMONE UPON OXYGEN CONSUMPTION OF CHICK OVARY SLICES¹

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URBANA

THE GONADOTROPHIC FUNCTION of the pituitary gland has been the subject of many investigations by endocrinologists but these studies have for the most part been limited to measurement of anatomical and gross physiological responses of the gonads. There has been little work done to elucidate the mechanism by which the pituitary gonadotrophic hormones produce these effects. It is known that the follicle stimulating hormone (FSH) promotes the growth of ovarian follicles but it is not known how FSH affects cellular processes to bring about this end result. Therefore, it seems of interest to measure responses of ovarian tissue to FSH at the cellular level. Since FSH stimulates the ovary to greater activity measurable as increase in weight and rate of function one might expect an increased rate of respiration of ovarian tissue in response to the hormone. In this study the effect of FSH on the oxygen consumption of chick ovary slices has been measured.

MATERIALS AND METHODS

The rate of oxygen uptake by chick ovary slices was measured by means of the Warburg apparatus. In most of the experiments White Leghorn chicks two to three months of age were used but in a few cases cross-bred chicks of a similar age were used. The animals were killed by luxating the cervical vertebrae. The ovaries were removed quickly, cleaned of the adhering adrenals and covering membranes, and placed in cold Krebs-Ringer solution buffered with phosphate to pH 7.2. The ovaries were sliced with a razor blade as quickly as possible, the lapse of time between killing of animals and taking of zero readings on the manometers being approximately one hour. The flasks containing the tissues were kept cold until they were placed in the water bath. Either four or six chicks were used in each run. The total number of animals used in the study was 351. If six flasks were used in a run ovaries from six chicks were used and the slices from these were distributed in such a way that each flask received a slice from each ovary and the slices in each

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flask were comparable in their position in the ovary (that is, each flask received a top slice, a second slice, a third, etc.). Manometer readings were taken at twenty-minute intervals for one hour. When manometric measurements were completed the tissues were removed from the flasks, dried overnight at 90°C., and weighed. The amount of tissue in each respiration flask was usually around 10 mg. dry weight. QO_2 was calculated as mm.³/mg. dry weight tissue/hour. Suspension media used were Krebs-Ringer solution buffered with phosphate to pH 7.2, and the same solution containing glucose in concentration of 0.02 M. or sodium pyruvate in concentration of 0.018 M. The respiration flasks contained 3 ml. of suspension medium in the main compartment and 0.2 ml. of 5% KOH in the center well. When hormone was added to the system the total dose was usually dissolved in 0.3 ml. of the suspension medium and this placed in the side-arm of the flask, the volume of the medium in the main compartment being reduced to 2.7 ml. The hormone solution was added to the main compartments of the flasks just before they were placed in the water bath. All measurements were made in an atmosphere of pure oxygen and at 40.5°C. which is approximately the body temperature of chickens. The biological activity of the hormones used in this study was determined on 21-day old female rats (Table 1).³

TABLE 1. BIOASSAY OF HORMONE PREPARATIONS

Prepn.	Total dose (mg.)	Time injected (days)	Rats		Av. wt. ovaries (mg.)	Response ovary
			Kind	No.		
FSH 5367	7.8	4.5	Normal	12	45	Follicles only
FSH 534	5.7	4.5	Normal	3	34	Follicles only
	8.7	4.5	Normal	6	59	Follicles only
FSH 96	3.4	4.5	Normal	6	30	Follicles only
	6.8	4.5	Normal	6	41	Follicles only
FSH 149	4.4	4.5	Normal	3	67	Follicles only
	8.8	4.5	Normal	3	84	Follicles only
FSH 4	3.2	4.5	Normal	3	41	Follicles only
	9.6	10.0	Hypophysectomized	6	130.5	Follicles only
	19.2	10.0	Hypophysectomized	8	192	Follicles only
FSH 126	11.2	4.5	Normal	6	29	Follicles only
FSH 535	8.1	4.5	Normal	6	34	Follicles only
F 116 A	0.67	4.5	Normal	19	81	Follicles few corpora
F 116 B	0.76	4.5	Normal	9	81	Follicles few corpora

RESULTS

Effect of *In Vitro* Addition of FSH.

Data obtained show (Table 2) that the addition of FSH to the medium in which chick ovary slices are suspended causes an increase in the rate of oxygen uptake by the tissue. This increase was demonstrated in three different media, being particularly marked in the

³ The hormone preparations and their bioassay values were supplied by Dr. W. H. McShan of the Department of Zoology, University of Wisconsin.

medium containing pyruvate. Comparison of "Control" values shows that addition of pyruvate to the buffered saline solution without hormone causes an increase of approximately 35% in QO_2 while comparison of "Test" values shows that the addition of pyruvate to the buffered saline solution containing FSH causes an increase of 60 to 80% in QO_2 . If the increased rate of oxygen consumption in the two cases can be regarded as a measure of oxidation of pyruvate it appears that FSH causes an increased rate of oxidation of this substance by ovarian tissue. Direct measurements of the utilization of pyruvate by ovarian tissue are being made in order to substantiate this conclusion. Addition of glucose to the medium without FSH has little effect on the QO_2 but with FSH present there is a definite increase on addition of glucose (Table 2).

The FSH preparations used in this study were not chemically pure. The sample used in the experiments reported in Table 2 was biologi-

TABLE 2. EFFECT OF ADDITION OF FSH IN VITRO ON RATE OF O_2 CONSUMPTION BY CHICK OVARY SLICES

FSH 5367 Amount of FSH added to flask (mg.)	QO_2 (Averages and standard deviations of 3 to 7 determinations)								
	Medium: Krebs-Ringer phosphate solution			Medium: 0.02 M. Glucose in Krebs-Ringer phosphate soln.			Medium: 0.018 M. Pyruvate in Krebs-Ringer phosphate soln.		
	Control	Test	% change	Control	Test	% change	Control	Test	% change
1	6.6 ± 0.6	7.2 ± 0.7	+9	7.2 ± 0.3	8.9 ± 0.8	+24	8.4 ± 0.4	11.5 ± 0.4	+37
2	7.0 ± 0.4	7.9 ± 1.1	+13*	7.4 ± 0.2	9.5 ± 0.9	+28	8.3 ± 0.4	12.6 ± 0.1	+52
3				7.2 ± 0.3	9.3 ± 0.9	+29	8.3 ± 0.4	13.2 ± 0.2	+60
4	7.0 ± 0.4	8.3 ± 0.3	+19	7.2 ± 0.3	10.0 ± 0.9	+39	8.4 ± 0.4	14.0 ± 0.8	+67
5	6.6 ± 0.6	7.7 ± 0.9	+17				8.4 ± 0.4	14.7 ± 0.8	+75
20	6.3 ± 0.3	8.7 ± 0.6	+38						

* Difference not statistically significant; all other differences between control and test in same medium significant ($p = .01$).

cally pure in the sense that it was free from other gonadotrophic hormones as judged by bioassay methods, but it was not a homogeneous substance. The question arises, then, whether the effects observed on addition of this substance to ovarian tissue are due to the hormone proper or to impurities present in the preparation. First of all, it was shown that the hormone preparation itself did not take up oxygen under the conditions of the experiments. FSH 5367 and FSH 534 were prepared from sheep pituitary glands by the method of McShan and Meyer (1940) in which FSH is separated from LH by digestion of the latter with trypsin. Substances which might be expected as impurities in such a preparation are products of tryptic digestion and possibly traces of trypsin itself. Of these only trypsin would be destroyed by boiling. The hormone being a protein would be expected to be denatured by boiling. Therefore, if a boiled solution fails to produce the effect that the same solution produces before boiling one can conclude that the effect is not due to the impurities which are unaffected by such treatment. In experiments to test the effect of a boiled preparation of FSH the hormone was dissolved in Krebs-Ringer phosphate

solution containing pyruvate and boiled for 20 minutes under reflux in a water bath. For control measurements untreated FSH was added to an aliquot of the medium which had received similar treatment. Results are shown in Table 3. Boiling of the preparation lowered its effect upon the rate of oxygen uptake by chick ovary slices. In the experiments using 5 and 20 mg. doses of FSH the effect disappeared completely in the boiled preparations. In the other cases there was a distinct lowering of effect but some activity remained. This residual activity may have been due to impurities present or to incomplete inactivation of the hormone.

TABLE 3. EFFECT OF BOILED AND OF DIALYZED PREPARATIONS OF FSH ON QO_2 OF CHICK OVARY SLICES. EFFECT OF GONADOTROPHIC HORMONE SAMPLES PREPARED WITHOUT TRYPTIC DIGESTION

Hormone	Amount added (mg.)	QO_2 (Medium: 0.018 M. Pyruvate in Krebs-Ringer phosphate)			
		No hormone	"Untreated" hormone	Boiled hormone	Dialyzed hormone
FSH 5367	5	9.9	11.5	10.0	11.2
FSH 5367	10	8.0	12.1	9.5	
FSH 5367	20	9.2	12.7	8.9	
FSH 534	10	8.4	13.1	9.7	
FSH 534	10	8.5	12.5	9.2	
FSH 534	10	8.6	14.4	9.1	
FSH 96	6	7.7*	12.1		
F 116 A	10	7.5 ± 0.5	8.9 ± 0.2		
F 116 B	10	$7.8 \pm 0.5^*$	10.2 ± 0.8		

* Ovaries from cross-bred chicks used in these determinations.

Data with FSH 5367, FSH 534, and FSH 96 are from single determinations.

Data with F 116 A and F 116 B are from two determinations (in duplicate) with each preparation. The differences with F 116 A and F 116 B are significant ($p = .01$).

Dialysis of a solution of the FSH preparation should remove any inorganic contaminants as well as products of tryptic digestion so this treatment would destroy the effect of the preparation on ovarian tissue if that effect were due to any of these impurities. Fifty milligrams of FSH 96 were dissolved in 4 ml. of distilled water and the solution placed in a dialyzing bag. This was placed in approximately 200 ml. of distilled water in a beaker and kept in the refrigerator for 24 hours, the water in the beaker being changed frequently during this period. The volume of liquid in the bag increased to about 5 ml. Six tenths milliliter of this solution per flask was used in the experiment. Control flasks with no hormone received 0.6 ml. of distilled water and control flasks with hormone received 0.6 ml. of distilled water containing 6 mg. of untreated FSH 96. Volumes in all flasks were made up to 3 ml. with Krebs-Ringer phosphate solution containing pyruvate. The dialyzed preparation caused an increase in the QO_2 of the tissue which was not quite as great as that produced by the non-dialyzed preparation (Table 3). The lowering of effect may have been

due to loss of some FSH activity since the hormone had been in solution for 24 hours. Maddock and Heller (1947) suggest that inactivation of solutions of pituitary gonadotrophins stored at icebox temperatures may occur, for the most part, during the first few days of storage.

The experiments with boiled and with dialyzed preparations of FSH samples do not rule out the possibility that the effect of preparations of FSH on the rate of oxygen consumption of ovarian tissue may be due to traces of trypsin. In Table 3 are shown the effects of two samples of hormones which were prepared by a method not involving the use of trypsin, F 116 A and F 116 B. Both of these increased the QO_2 of chick ovary slices. From the results of the experiments reported in Table 3 it appears that the increase in QO_2 of ovarian tissue caused by addition of the FSH preparations is due to the hormone and not to impurities present in the preparations.

The possibility that the increase in QO_2 is a non-specific protein effect was tested by adding other proteins of pituitary origin to the system containing the tissue slices. The adrenocorticotrophic hormone and the luteinizing hormone were chosen for this purpose. Five determinations were made measuring the QO_2 of chick ovary slices in the pyruvate medium with no hormone and with 5 mg. doses of ACTH, LH, and FSH. Neither ACTH nor LH affected the rate of oxygen uptake by the tissue, the average QO_2 values being 7.9 with ACTH and 8.9 with LH as compared to 8.1 with no hormone. In contrast FSH caused a highly significant increase producing an average QO_2 value of 11.1 ($p = .01$). The slight though not significant increase obtained with LH may be suggestive since LH might be expected to have a trophic effect on the interstitial (androgen-producing) cells of the ovary.

An experiment was performed to determine whether the magnitude of the increase in QO_2 of chick ovary slices produced by FSH preparations *in vitro* is correlated with the gonadotrophic potency of the preparations as judged by the increase in weight of ovaries of immature rats caused by injection of these preparations. By such bioassay FSH 4 was found to be considerably more active than FSH 535 and FSH 126. The latter two preparations were not greatly different in activity though FSH 535 was somewhat more potent (see Table 1). These three preparations were tested for their effect upon the QO_2 of chick ovary slices in the pyruvate medium. Five milligram doses of the hormones were used. The average QO_2 values of three determinations were: no hormone, 8.3; FSH 4, 10.3; FSH 126, 9.9; FSH 535, 8.4. The increases produced by FSH 4 and FSH 126 are significant but they are not significantly different from each other. FSH 535 in a 5 mg. dose did not produce a significant increase in QO_2 of the ovary slices. Thus, no correlation between gonadotrophic potency

as determined by bioassay and the magnitude of the effect on QO_2 of chick ovary slices was demonstrated by this experiment.

In Fig. 1 are plotted the data from Table 2 showing the effect on QO_2 of chick ovarian tissue of varying the concentration of FSH in the medium in which ovary slices are suspended. The concentration of the hormone within the cells of the tissue is probably not identical with the concentration in the medium but rather a function of it. It can be seen that the effect on QO_2 increases with concentration of the hormone up to a certain level and that the concentration which produces the maximum effect is lower in the medium containing

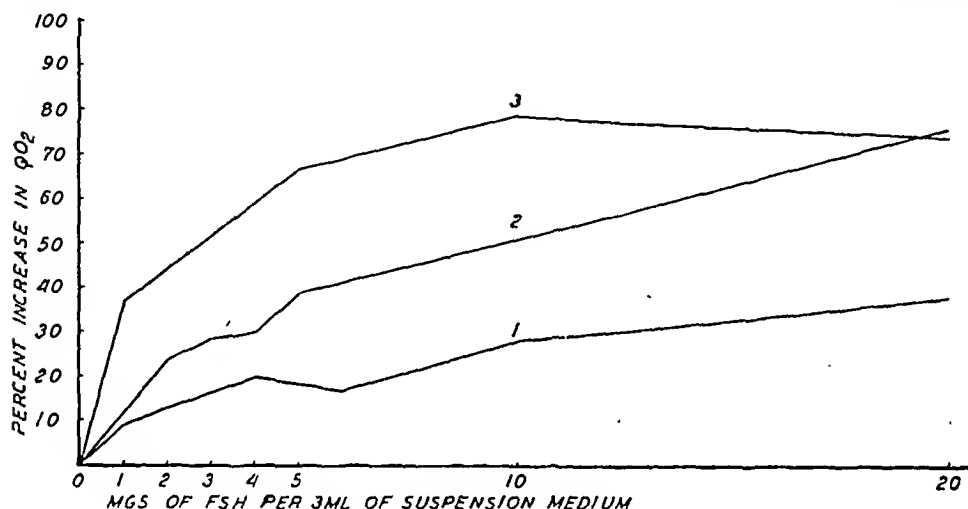


FIG. 1. Effect of Varying Amounts of FSH Added *in Vitro* on the QO_2 of Chick Ovary Slices.

1. Tissue suspended in Krebs-Ringer phosphate solution
2. Tissue suspended in Krebs-Ringer phosphate solution plus glucose
3. Tissue suspended in Krebs-Ringer phosphate solution plus pyruvate

pyruvate than in the other media. With glucose the maximum effect may be reached at a lower concentration than is indicated on the curve since no measurements were made at the 10 mg. level. The point at the 20 mg. level is based on only two observations (not included in Table 2). The points at the 10 mg. level on the other two curves are also based on two observations in each case.

In Table 4 are shown the results of experiments in which the rate of oxygen consumption of ovary slices from chicks that had received injections of FSH was compared with that of ovary slices from untreated control chicks. In the first two experiments test animals received subcutaneous injections of FSH daily for 10 days and were killed on the eleventh day for the respiration measurements. There was no significant difference between respiratory rates of the ovaries of these birds and those of untreated controls. Since exogenous FSH is probably rapidly inactivated by the liver it seemed likely that the 24-hour interval between the time of the last injection and the time of

killing allowed ample time for the FSH concentration of the blood and the ovaries of the treated animals to return to the endogenous level. So a third experiment was performed in which the test animals were given a large dose of FSH in a single intravenous injection and were killed 5 to 15 minutes later. In these the FSH level in the ovary should have been higher than that of the untreated controls. The QO_2 measurements were made in the pyruvate medium only. There was found to be an increase due to the injection of FSH which though small was highly significant ($p = .01$).

TABLE 4. EFFECT OF INJECTION OF FSH INTO CHICKS ON RATE OF O_2 CONSUMPTION BY OVARY SLICES

FSH	Total dose (mg.)	Injection period and site	Time lapse between last injection and killing of animal	QO_2 (Averages and standard deviations of 4 to 5 determinations)					
				Control			Test		
				Krebs-Ringer phosphate	0.02 M. Glucose in KRP	0.018 M. Pyruvate in KRP	Krebs-Ringer phosphate	0.02 M. Glucose in KRP	0.018 M. Pyruvate in KRP
96	34	Daily for 10 days S.C.	24 hrs.	6.8±0.4	7.1±0.5	8.2±0.2	6.8±0.4	7.3±0.4	8.7±0.5*
149	26	Daily for 10 days S.C.	24 hrs.	6.2±0.3	6.7±0.6	8.3±0.5	6.6±0.5	6.6±0.3	8.6±0.6*
96	100	Single Injection I.V.	5 to 15 min.			5.0±0.7			9.0±0.6†

* Differences between comparable QO_2 values of control ovaries and test ovaries not significant.

† Difference between QO_2 of control ovaries and test ovaries significant ($p = .01$).

DISCUSSION

From the results of the experiments reported here it appears that FSH affects some enzyme system or systems involved in oxidative mechanisms in ovarian tissue. Whether the effect is produced by direct participation in such a system, by release of some physiological inhibitor of it, or by some other mode of activation cannot be said, but the magnitude of the effect seems to depend upon the concentration of the hormone present in the tissue. This is apparent both from results of experiments varying the amount of FSH added to the medium in which ovary slices are suspended and the results of the experiments in which test animals received FSH by injection. The latter experiments indicate that the presence of the hormone in the tissue at a given time is necessary for its effect on oxygen consumption, that there is not an initial stimulation by FSH which is sustained after the hormone level drops.

In a review of the literature on the development of the Graffian follicle Hisaw (1947) points out that follicular growth can proceed in mammals to the stage of antrum formation in the absence of the pituitary gland and that it is only as the animal approaches sexual maturity that its ovaries become responsive to pituitary gonadotrophins, while the ovaries of the very young animal are refractory. Furthermore, quoting from the same review, "it is also a common observation that even in the adult animal all the follicles are not equally

responsive. When a pituitary extract is given in small doses to a rat the effects may be limited almost entirely to the larger follicles, but if the dosage is increased smaller follicles are called into the reaction, while the primordial follicles fail to be stimulated even when large amounts of the preparation are injected." There is a long period in the chick (150 days) in which the ovary does not respond to administered mammalian FSH with increase in weight of the organ as the criterion of response (Nalbandov and Card, 1946). It is interesting that the ovaries in which the increase in QO_2 due to FSH was observed were from chicks of this "refractory" age. Because of this observation it seems possible that the initial effect of FSH (and possibly the only effect) is the activation of oxidative mechanisms in the ovary thereby making an increased amount of energy available to the organ for developmental processes and that the lack of the end response, growth, in the ovary of the very young animal is due not to lack of response to the hormone but to some limiting factor in the subsequent chain of events leading to growth of the organ. Is it not possible that the mechanism for development of the ovary to full maturity is present in the organ itself and is controlled by the pituitary hormones in only a quantitative way by regulation of the energy available for its function? Such a concept would explain the lack of response to administered FSH of the ovary of the very young animal if we assume an orderly pattern of development of the organ regulated by endogenous mechanisms for which certain quanta of energy are required. Increasing the available energy before it is needed would produce no change in the pattern. On the other hand, lack of the pituitary hormones when the organ has reached the stage where a greater amount of energy is required would disrupt the pattern.

SUMMARY

The addition of FSH to the medium in which chick ovary slices are suspended causes an increase in the rate of oxygen uptake by the tissue. The magnitude of the response to FSH *in vitro* depends upon the concentration of the hormone in the medium as well as upon the substrate added to the medium.

The effect of FSH on the QO_2 of chick ovary slices is destroyed or lowered markedly in boiled preparations of the hormone, but is lowered only slightly in a dialyzed preparation, indicating that the effect is produced by the hormone rather than by impurities in the preparation. That the effect is not due to a non-specific protein action is shown by the fact that neither ACTH nor LH added to the suspension medium affects the rate of oxygen consumption of chick ovary slices.

The QO_2 of slices of ovaries from chicks that had received daily subcutaneous injections of FSH with a 24-hour lapse of time between the last injection and measurement of the respiratory rate of the ovaries was not significantly different from that of ovaries of un-

treated control animals. A single intravenous injection of FSH with an interval of 5 to 15 minutes between the time of injection and that of killing of animals for manometric measurements caused a significant increase in the QO_2 of the ovary in a medium containing pyruvate as compared to the QO_2 in the same medium of ovaries of untreated control chicks.

The possible significance of these findings with respect to the mechanism of action of FSH is discussed.

ACKNOWLEDGMENT

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NOTES AND COMMENTS

FAILURE OF ADRENOCORTICAL EXTRACT TO MODIFY THE IMMUNITY ACQUIRED BY INTACT MICE THROUGH THE USE OF PNEUMOCOCCAL VACCINE¹

The low resistance of Addisonian patients and adrenalectomized animals to infection has led to speculation that adrenal function may influence the processes concerned with acquired immunity (Perla and Marmorsten, 1941). In support of this view two groups of investigators (Fox and Whitehead, 1936; Chase, White and Dougherty, 1946) have presented data indicating increased antibody formation in intact animals treated simultaneously with antigens and adrenocortical extracts. Since these results were not confirmed by other investigators (Eisen, Mayer, Moore, Tarr and Stoerck, 1947; Murphy and Sturm, 1947; Thatcher, Houghton and Zeigler, 1948) it was thought that another approach, independent of *in vitro* antibody determinations, might yield pertinent information.

METHODS AND PROCEDURES

The method chosen was to test the resistance of mice to active pneumococcal infection, after treatment with adrenocortical extracts and appropriate antigens, upon the assumption that any general increase in antibody would include an increase in specific protective substances. White Swiss mice weighing 18–22 grams were immunized with intraperitoneal injections of heat-killed Type I Pneumococci, and challenged 4–14 days later with living cultures of the same organism. Undiluted broth cultures contained approximately two billion diplococci per cc.; the bacteriological procedures have been described elsewhere (Vollmer and Samsell, 1949). Adrenocortical extract (ACE)² was administered subcutaneously in unitary doses of 0.5 cc., a quantity reported to produce lymphopenia in mice (Dougherty and White, 1944) or of 0.2 cc., which in repeated doses with antigens has been reported to enhance the antibody titer (Chase, White and Dougherty, 1946). As a control for aqueous ACE, which contained 10 per cent ethanol, isotonic saline solution containing 10 per cent ethanol was used.

RESULTS

Treatment of previously immunized mice with ACE given shortly before infection: Preliminary trials had shown that when a group of mice had been immunized with a given dose of killed pneumococci, 60–90 per cent of them would die after injection with the same dose of living pneumococci ("equivalent dosage"). This relationship was utilized in an attempt to determine

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¹ The authors assume full responsibility for the statements made in this report, which in no way reflects official naval opinion.

² Adrenal Cortex Extract (Upjohn), equivalent to 0.2 mg. 11-dehydro-17 hydroxycorticosterone per cc.

whether the injection of ACE in immunized mice just before infection would alter their mortality rate. One hundred twenty mice (60 males, 60 females) were injected with 0.05 cc. of a 10^{-3} dilution of standard vaccine. Two weeks later they were separated into two equal groups and injected with 0.5 cc. of ACE or saline. One-half hour later all were challenged with 0.5 cc. of a 10^{-4} dilution of a viable culture (equivalent dose). Only three controls and five hormone-treated mice survived.

Attempt to increase protection with one or two treatments of ACE at time of immunization: In a parallel experiment 120 mice were immunized with killed pneumococci and challenged four days later. In this instance the extract or

TABLE 1. SURVIVAL OF MICE IMMUNIZED WITH SIX INJECTIONS OF VACCINE WITH SIMULTANEOUS INJECTIONS OF ADRENOCORTICAL EXTRACT OR CONTROL SOLUTIONS, AND CHALLENGED WITH LIVING PNEUMOCOCCI

Vaccine dilution (0.05 cc.)		Number of survivors per group of six mice, challenged with Inocula (0.5 cc.) in series of three-fold dilutions, beginning with undiluted broth culture (A)										Survivors
		A	B	C	D	E	F	G	H	I	J	
10^{-2}	Exp.	0	0	0	1	4	4	6	6	6	6	33/60
	Con.	0	0	0	2	3	5	5	6	5	6	32/60
10^{-3}	Exp.	0	0	0	0	0	1	1	2	5	6	15/60
	Con.	0*	0	0	0	0	2	1	4	3	6	16/59
Survivors	Exp.	0	0	0	1	4	5	7	8	11	12	48/120
	Con.	0	0	0	2	3	7	6	10	8	12	48/119

* Five mice in this group.

control solution was administered at the same time as the immunizing vaccine. Again, the difference between the terminal mortality rates was very small, 20 controls and 22 treated animals surviving the injection.

Two similar experiments, with the conditions altered slightly, were carried out with similar results. In the first, a merthiolate-preserved adrenocortical extract³ was administered at the same time as the immunizing vaccine. In the second, two doses of the alcohol-preserved extract were administered, one an hour before the immunizing vaccine and the other three hours later. Upon challenge with live pneumococci 63 (of 84) and 56 (of 90) mice died in the extract-treated groups, and 68 (of 84) and 51 (of 90) died respectively in the control groups.

Treatment with ACE at time of immunization, with varied protective and challenging doses: Each of 84 mice was given 0.5 cc. of ACE and simultaneously inoculated with one of four ten-fold dilutions of vaccine. They were later challenged, along with 84 controls, with inocula of bacteria of five different dilutions. The treated and control animals had the same pooled mortality rate (47/84) and similar mortality rates in their corresponding subgroups.

Repeated treatment with ACE and vaccine, simultaneously administered:

³ Adrenal Cortex Extract (Wilson), equivalent to 75 gm. of fresh adrenocortical tissue per cc. This extract contains sodium ethyl mercuric thiosalicylate (1/100,000) and the control mice were therefore treated with isotonic saline containing the same amount of this preservative.

120 male mice were treated with 0.2 cc. of ACE at the time of each of six successive doses of vaccine (two weekly for three weeks), and 120 controls were treated with 0.2 cc. of saline solution and the vaccine. One week after the last treatment the mice were challenged with inocula of pneumococci, in a series of threefold dilutions. The pooled mortality rates of the experimental groups and their controls were practically identical, and the distribution of deaths was essentially the same throughout the sub-groups (table 1).

COMMENT AND CONCLUSIONS

It was evident throughout that the degree of immunity acquired by groups of mice varied according to the immunizing and challenging doses of bacteria administered, but was not materially affected by their treatment with ACE. This finding is consistent with that of Eisen and colleagues (Eisen, Mayer, Moore, Tarr and Stoerck, 1947) that the use of ACE did not increase the formation of precipitins to type I pneumococcal polysaccharide in adrenalectomized rats, and tends to discount the possibility raised by the work of Chase (Chase, White and Dougherty, 1946) that in one of the adrenocortical substances a nonspecific physiological adjuvant to immune response might have been found. However, the endocrine and bacteriological variables of this field have not been adequately studied for the presence of modifying factors. Therefore while the present experiments appear to indicate that whole ACE may not stimulate any extra reaction to antigens in intact animals, they should not be interpreted to exclude the possibility of indirect effects upon the immunity response of animals under the stress imposed by active infection or by different experimental conditions.

SUMMARY

In order to ascertain whether or not adrenocortical hormones increase the immune response of intact animals to antigens, white Swiss mice were immunized with Type I pneumococcal vaccine and simultaneously treated with ACE or control injections. They were later challenged with living pneumococci of the same strain. Dosages of vaccine and challenging inocula were varied over a wide range. In all instances the results were negative, indicating that whole adrenocortical extract administered early in the immunization period did not modify the resistance of the animals to active homologous infection.

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GROWTH HORMONE AND FAT METABOLISM

IN A FURTHER STUDY of the possible identity of urinary adipokinin (Weil & Stetten, 1947) and a pituitary factor, the effect of purified growth hormone upon the fat content of the mouse liver has been studied. Purified growth hormone¹ was injected hypodermically into fasting mice and the fat content of their livers determined by methods previously described. In confirmation of observations of others (Szego & White, 1948, 1949), a striking increase in total liver lipids was found to follow the administration of as little as 100 μ g. of hormone (Table 1), a doubling of the control value occurring after 7 hours.

TABLE 1. FEMALE MICE, FASTED FOR 7 HOURS, INJECTED SUBCUTANEOUSLY WITH 100 G GROWTH HORMONE PREPARATION, 3PKR3 ARMOUR & CO., IN 0.5 CC SALINE, 2, 4, AND 7 HOURS BEFORE SACRIFICE. CONTROLS INJECTED WITH SALINE 7 HOURS BEFORE SACRIFICE

Time interval	Number of mice	Weight of mice	Mean liver weight	Mean liver fat per 100 g. body weight
2 hours	16	21, 5-24	1, 61	413 \pm 19*
4 hours	12	22-24	1, 88	564 \pm 13
7 hours	16	21, 5-24	1, 91	685 \pm 15
controls	30	20-26	1, 66	326 \pm 11

* Standard error: $\sqrt{\frac{\sum(v^2)}{n(n-1)}}$

In an analysis of the mechanism of action of growth hormone, it is of interest to establish the chronological sequence of events which follows its administration. In this regard we consider it worthy of note that a statistically significant increase in the quantity of liver fat is seen within 2 hours of the administration of this hormone. Thus the adipokinetic action of growth hormone appears to occur simultaneously with its effect upon the level of blood amino acids which has been demonstrated 1 to 2 hours after administration of this hormone to rats (Russell & Capiello, 1949).

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GOITEROUS CHICKS FROM IODINE-INJECTED EGGS¹

WOLFF AND CHAIKOFF (1948 a, b, c) have shown that administration of large amounts of inorganic iodide inhibits synthesis of thyroxine in the normal thyroid gland. However, this inhibition is temporary unless nephrectomized animals are used to maintain the high blood level of inorganic iodine. It would appear that the developing chick embryo might also be an excellent subject for study of the effects of prolonged thyroid inhibition. The constant supply of iodine provided by injecting inorganic iodine into the white of fertile eggs should be somewhat comparable in effect to a single dose administered to a nephrectomized animal.

Prolonged inhibition of thyroid activity would be expected to result in enlargement of the gland as a result of increased secretion of thyrotropin by the pituitary. It is speculated that such an effect might be involved in the production of goiters in chicks hatched from eggs laid by hens fed diets containing synthetic thyroprotein (STP), especially since STP contains from 1.5-2.0 per cent inorganic iodine as iodide (Graham 1948). The present communication reports a preliminary test of this hypothesis.

EXPERIMENTAL

Logarithmic doses of inorganic iodine as KI in sterile distilled water were injected into the white of fertile eggs on the 16th day of incubation. On the day of hatch the chicks were exsanguinated by decapitation and the thyroids were immediately excised and weighed on a Roller-Smith balance.

Levels of inorganic iodide from 156μ to $40,000\mu$ per egg resulted in progressive and significant thyroid enlargement; dosages from 0.6μ to 39μ failed to produce goiter (Table 1). At the dosage levels which produced goiters incubation time was increased and the navels of the chicks were incompletely closed. Embryonic mortality was apparently greater at the higher levels but the numbers in each group are not sufficiently large to justify a positive statement.

TABLE 1. GOITEROGENIC INFLUENCE OF INORGANIC IODIDE (AS KI) WHEN INJECTED INTO FERTILE EGGS ON THE 16TH DAY OF INCUBATION

Amt. I inj. in each egg	No. chicks hatched ¹	Mean thyroid weight at hatch	Ratio to control
(gamma)		(mg \pm SE)	
40,000	3	10.0 ± 0.34	3.23
10,000	4	9.6 ± 0.82	3.10
2,500	8	8.9 ± 0.58	2.90
625	9	7.3 ± 0.52	2.35
156	8	5.8 ± 0.52	1.90
39	7	4.3 ± 0.46	1.40
9.8	6	3.8 ± 0.38	1.20
2.5	9	4.4 ± 0.38	1.40
0.6	9	3.8 ± 0.30	1.20
Control	9	3.1 ± 0.13	—

¹ Ten fertile eggs were injected with each dosage, but not all the chicks hatched.

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¹ This study was made possible through funds made available jointly by the Carnegie Foundation and the University of Georgia. The writers, however, are solely responsible for the statements made in this report.

COMMENT

In earlier studies the present writers have shown a quantitative relationship between level of STP fed to the hen and increase in incubation time and degree of thyroid enlargement of their chicks (Wheeler and Hoffmann, 1948 a, b). An hypothesis that this effect was due to reduced maternal thyroid activity with an attending failure of the thyroïdal substance to be deposited in the egg proved untenable when the feeding of desiccated thyroid to hens failed to alter incubation time or thyroid size of their chicks (unpublished data). Since the iodine content of the hen's diet determines the iodine content of the egg (Wilder, Bethke and Record, 1933), the work of Wolff and Chaikoff provides an alternative hypothesis in that the inorganic iodide present in the STP fed to hens might increase the iodine content of the egg sufficiently to inhibit thyroxin synthesis by the embryonic chick thyroid. This in turn would allow increased thyrotropin secretion and thyroid enlargement. Such a mechanism would adequately explain the goiters found in newly hatched chicks as well as their hypothyroid symptoms such as the lowered oxygen consumption rates observed by McCartney and Shaffner (1949) and poor closure of the navel.²

It may be appropriate to point out that although the injection of STP into eggs was comparable in its effect to the injection of an equivalent amount of thyroxine (Booker and Sturkie, 1949), the validity of the present data is in no way impaired because the simultaneous injection of thyroxine with iodine (both of which are present in STP) would inhibit the release of thyrotropin by the pituitary.

The present findings show that injected inorganic iodide will produce goiter in chicks and increase incubation time. Work is now in progress to test whether the feeding of inorganic iodide to hens will also result in the production of goiterous chicks.

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² Poor closure of the navel has been established as a symptom of hypothyroidism in the progeny of pigeons made hypothyroid as a result of iodine deficiency (Hollander and Riddle, 1946), and in chicks from thiourea-injected eggs (Grossowicz, 1946).

³ Our indebtedness to Mr. John Brooke for technical assistance is gratefully acknowledged.

ASSOCIATION NOTICE

ANNOUNCEMENT

The membership roll of the Association is being revised in preparation for the issuance of a new Roster as of December 31, 1949. Copy for this must be in the hands of the printer not later than October 1, 1949.

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HENRY H. TURNER, M.D.
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EFFECTS OF CASTRATION AND SUBSEQUENT ANDROGEN ADMINISTRATION UPON MATING BEHAVIOR IN THE MALE HAMSTER (*CRICETUS AURATUS*)¹

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INTRODUCTION

EXPERIMENTAL investigations and clinical observations have yielded a great deal of evidence concerning the effects of castration upon the sexual capacity of male mammals. The sequelae to adult castration in human beings are ambiguous. Some patients describe pronounced and fairly prompt reduction in erotic responsiveness and capacity for sexual performance. Other castrated men claim that normal libido and potency are retained for several decades (Lange 1934, Daniels and Tauber 1941). This extreme variability might be due to any one of several causes. (1) It is possible that individuals vary endocrinologically, and that extragonadal androgens are in some cases sufficient to support an essentially normal sex life. (2) Claims for continued virility may be false. (3) The patient's expectations and desires may exert a powerful control over his sexual capacities with the result that castration provides a welcome excuse for inactivity or, in contrast, that hormonal deficiencies are compensated by increased "psychic" (cerebral) stimulation.

To weight the various possibilities would indeed be difficult and in any event quite outside the province of this report. More objective

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and reliable evidence on the behavioral consequences of gonadectomy comes from studies of subhuman mammals.

One prepuberally castrated male chimpanzee has been under more or less constant observation for a number of years since the operation. During childhood and adolescence sexual development was normal. Masturbation occurred and sex play was noted. After attaining adult status this prepuberal castrate mated readily and vigorously with receptive females. Save for the absence of ejaculation his behavior was identical with that of an intact and experienced breeder (Clark 1945).

The postcastrational survival of mating behavior in male mammals is not limited to man and the other anthropoids. Evidence dealing with carnivores is scanty but incidental observations made by the senior author suggest that some male cats castrated during adolescence continue to show sexual responses including genital reflexes and copulatory thrusts at least six years after the operation. A study now being conducted by the senior author indicates that there is no marked reduction in the mating performance of male dogs during the first year after adult castration, and subsequent tests of these animals may show that sexual capacity is retained indefinitely.

The consequences of castration in rodents and lagomorphs are quite different. The majority of experimental studies on this subject have dealt with rats, guinea pigs and rabbits and the results are fairly consistent from species to species. Gonadectomy in adulthood is followed by fairly rapid decline in sexual responsiveness. Ejaculation is the first reaction to disappear, and the capacity for erection and intromission is greatly reduced within two or three weeks after operation. By the second postoperative month mating behavior has reached a very low level at which it remains thereafter. This does not, however, represent complete abolition of sexual excitability. In the case of the rat incomplete copulatory responses occur occasionally for many months after testicular loss.

The apparent difference in the effects of castration upon rodents and lagomorphs as contrasted with carnivores and primates raises the interesting question as to whether the dependence of sexual behavior upon androgen is in some way related to phylogenetic status: The experiment to be described here dealt with a rodent species belonging to a genus in which these problems have not been investigated. The golden hamster was chosen as an experimental subject not only because of its systematic position but also because its mating pattern differs in several respects from that of the rat and the guinea pig.

SUBJECTS AND METHODS

The experimental subjects were 19 young adult male hamsters approximately two months of age at the beginning of the observational period. In this species fertile mating begins during the sixth or seventh week after birth (Bond 1945, Reed and Reed 1946). Each male was kept in an individual

cage and all of them lived in a colony room which was darkened from 7:00 A.M. to 7:00 P.M. and well-lighted the remaining 12 hours. This reversal of the light-dark rhythm caused the stimulus females to come into estrus during the day. The animals were fed a diet of lettuce and dog chow supplemented once a week with fresh horse meat. The females used as stimulus animals were the same age as the males and were maintained under similar conditions.

Reed and Reed (1946) have described the copulatory behavior of this species in some detail and our observations agree with theirs in all important particulars. However, instead of adopting the terminology used by these writers we have employed a slightly different one developed in connection with studies of rats, guinea pigs and rabbits. This procedure facilitates inter-species comparisons.

The receptive female flattens her back and stands quietly, allowing the male to mount. Grasping his partner about the flanks the male straightens his hind legs and raises the penis as high as possible, making several rapid, piston-like pelvic thrusts. He may dismount after thrusting several times. Reed and Reed refer to this response as "phase 1" of a copulation. Here it will be termed an abortive or incomplete copulation for reasons to be considered below.

Upon other occasions the male concludes a series of thrusts with a deeper and more vigorous movement in which his pelvis is brought firmly against and slightly beneath that of the female. Then instead of dismounting at once, the male continues to press tightly against the female for 2 or 3 seconds. Reed and Reed named this reaction "phase 2" and stated that it signalled a "successful copulation." We observed hamsters while they mated in a glass-bottomed cage set above a mirror and found that the deep and prolonged final thrust accompanies insertion of the penis, whereas the "phase 1" response never includes intromission. The terms "complete copulation" or "intromission" are therefore used here to indicate the pattern referred to as "phase 2" in Reed and Reed's report.

The earlier workers were able to determine with "reasonable accuracy" the occurrence of emission or ejaculation by observing the temporal spacing of successive mounting reactions and the intervening rest periods. Our animals did not behave in the manner described by Reed and Reed and we were forced to conclude that any relation between ejaculation and observable behavior was too variable to be relied upon.

Before each day's tests receptive females were chosen on the basis of their response to a sexually active "indicator male." The experimental male was put in a small glass aquarium and left there for at least five minutes in order that he might become adapted to the environmental situation. Preliminary observations showed that even when a receptive female was present a male was likely to spend the first few minutes investigating the environment. Males that were allowed to carry out this exploration before the test began tended to initiate mating responses promptly as soon as the female was presented.

At the end of the adaptation period a receptive female was introduced and a timer was started. If no intromissions were achieved within ten minutes after the female's entrance the test was scored as negative. If the male did effect intromission in 10 minutes or less observations were continued for 15

minutes from the time of the first intromission. As the test proceeded the frequency of intromissions and abortive copulations was recorded and notes on other aspects of the males' performance were made.

Each male was given 10 sex tests before castration, the tests being spaced at intervals ranging from 3 to 21 days. Analysis of the results indicated that within these limits the length of the rest period between tests has no effect upon sexual behavior. The experimental population was divided into five groups equated as nearly as possible in terms of average frequency of intro-

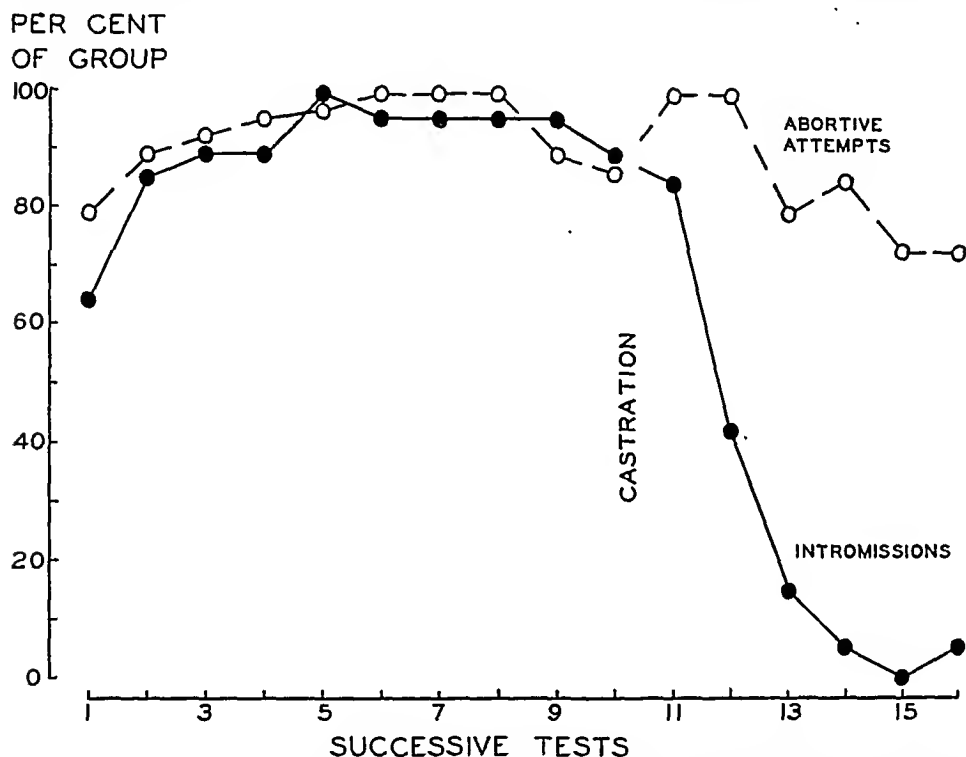


FIG. 1. Percentage of the group achieving at least one intromission in each test before and after castration (solid line) and percentage executing at least one mount without intromission (broken line).

missions per preoperative test. One group was first tested on the third day after castration, another on the fourth day and one each of the remaining castrate groups received their initial postoperative tests on the fifth, sixth and seventh days after gonadectomy. Thereafter all animals were tested once a week for six weeks. Analysis of results failed to show any reliable change in sexual performance for intervals of less than a week and therefore in preparing this report the postoperative records of the five subgroups have been combined.

Three months after castration, and thus six weeks after the sixth post-castrational mating test, one brief test was given and daily injections of testosterone propionate were begun.² One group of castrates received 1 microgram (.001 mg) of androgen daily for a period of two weeks. Since this

² The testosterone propionate employed in these experiments was Oreton generously supplied by Dr. Edward Henderson of Schering Corporation, Bloomfield, N. J.

dosage had no apparent effect upon sexual behavior the concentration was raised to 100 micrograms per 24 hours and sex tests were continued. A second group of castrates was treated with 75 micrograms per day from the beginning of the injection period.

At the beginning of the third week of the injection period daily sex tests were conducted and these continued until the complete copulatory response (intromission) had reappeared. If, during his first positive test, a particular male performed as well as he had before operation his tests were considered completed and he was dropped from the experiment. If, during the test in which intromission first reappeared it was performed only a few times, the male was allowed a week's rest, daily injections were continued and additional sex tests were conducted at the rate of one per week. The weekly tests were continued until the male had been restored to preoperative potency or until it became necessary to terminate the experiment.

RESULTS

Number of males achieving intromission

The solid line in figure 1 shows the proportion of the group achieving one or more intromissions in tests conducted before and after castration. In the course of the first few tests there was some increase in the number of males responding, but during each of the last 5 preoperative tests from 90 to 100 per cent of the group displayed this response at least once. Within two weeks after castration the number of animals achieving intromission was greatly reduced. As the postoperative interval lengthened the group performance continued to decline until during the fifth week after castration none of the 19 males effected intromission.

Power of intromission was not completely lost, however, for one week later two males achieved intromission once or twice; and in a brief test given three months after castration this reaction was shown a limited number of times by two other individuals. It is possible that some slight capacity for intromission may survive indefinitely despite the absence of testicular hormone.

Frequency of intromissions per male per test

The actual frequency with which intromission is achieved by normal male hamsters is subject to considerable individual variation. Before operation our animals showed a range of 1 to 85 intromissions in separate tests and the average frequency per preoperative test ranged from 18 to 73 for different males. These averages are based upon only those tests in which intromission occurred at least once. Despite the wide variability of individual performance, the intromission score in successive tests proved to be reasonably consistent for any given male. The odd-even reliability was $+.89 \pm .03$.

During the first four days after removal of the testes there was no significant reduction in the frequency of intromission, but with longer postoperative intervals this response decreased markedly. The solid

line in figure 2 shows the average frequency of intromission during those tests in which this reaction appeared at least once. As seen in figure 1 the number of males continuing to achieve intromission was rapidly reduced during the first few postoperative weeks; and figure 2 reveals that even when this response survived it occurred less and less frequently in successive postoperative tests. Before operation the average intromission frequency ranged from 34 to 52 in different tests, but those males which succeeded in effecting intromission during the test given one month after castration did so an average of only 13

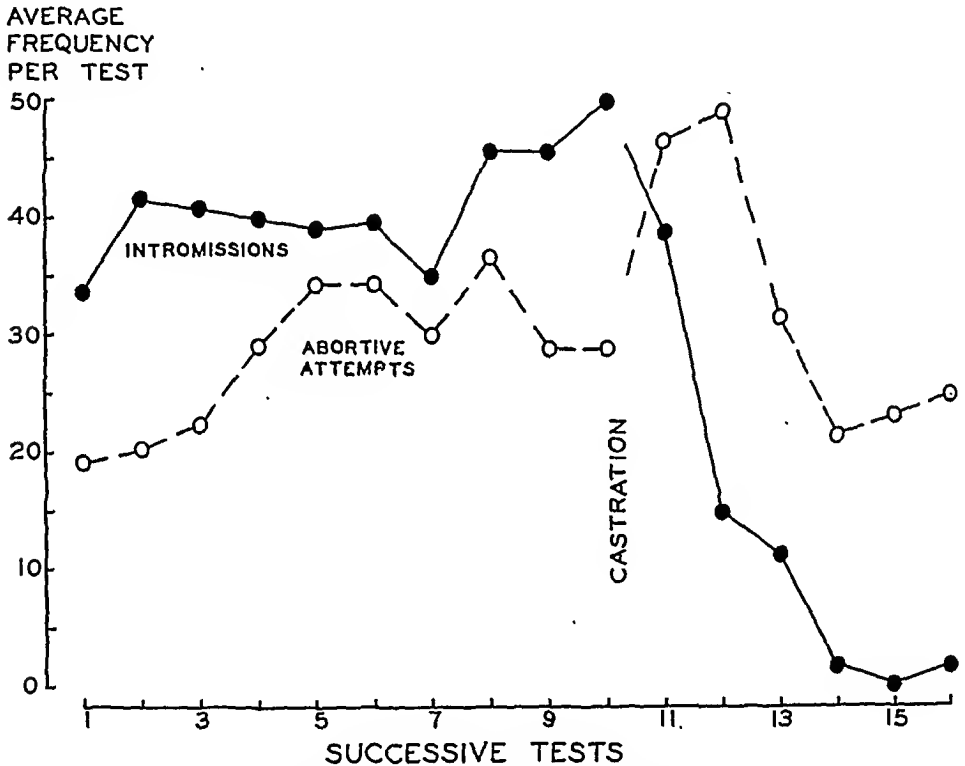


FIG. 2. Average frequency of intromissions per male per test before and after castration (solid line) and average frequency of mounts without intromission (broken line).

times. Including all postoperative tests in which intromission occurred, the average frequency was found to be 32.2 responses per test as compared with a preoperative average of 43.1. This difference is highly significant according to Student's *t* test ($P = .01$).

It is not clear why some castrates retained copulatory activity longer than others but there is some suggestion of a positive relationship between pre- and postoperative sexual vigor. The experimental subjects were ranked according to the greatest number of intromissions achieved in any one test after operation, and they were also ranked according to average intromission frequency in preoperative tests. The coefficient of correlation between these two rankings was $+0.71 \pm 0.08$.

With respect to the effects of castration upon the intromission response three generalizations are indicated. (1) The number of males achieving intromission declined rapidly in successive weeks after the operation and after approximately one month less than 10 per cent of the group responded in any single test. (2) Those males that did exhibit intromission postoperatively were able to do so only a limited number of times. The actual frequency of this reaction dropped off rapidly and by the end of the first postoperative month the few individuals capable of responding effected only two or three intromissions during a 15 minute period with the receptive female. (3) Our results suggest that some slight capacity for intromission survives in at least some individuals as long as three months after removal of the testes.

Number of males showing abortive attempts

The broken line in figure 1 shows the per cent of the experimental group exhibiting at least one abortive or incomplete copulatory attempt in each of the tests. The proportion of the group responding increased during the first few tests and ranged between 90 and 100 per cent for the last seven preoperative tests. Following castration this measure of sexual activity showed little change for the first two weeks, and even six weeks after operation three-quarters of the males made abortive attempts to copulate. During a brief test given three months after castration 14 of the 19 males made one or more of these responses.

Differences between the two graphs in figure 1 suggest that the tendency to mate survives longer after castration than does the ability to achieve intromission. It seems likely that hormonal deficiency leads to failure of erection despite the fact that the remaining sexual excitability is sufficient to call forth numerous copulatory attempts.

Frequency of attempts per male per test

Before operation the experimental males frequently mounted the female without achieving intromission. The average scores per test ranged from 12.1 to 41.5 for different individuals. Test-to-test consistency was fairly high and the odd-even reliability coefficient was $+.76 \pm .07$. The broken line in figure 2 shows the average frequency of abortive copulations in each successive test. For the first two weeks after castration the number of incomplete contacts increased well above preoperative averages. Thereafter the frequency of this response returned to preoperative levels and showed no further decline during the test period. It seems clear that castration did not eliminate mating tendencies although it did greatly reduce the probability of completed copulations with intromission.

Initial latencies

The initial latency was the number of seconds elapsing between the introduction of the receptive female and the male's first intromission. In preoperative tests this function showed a low negative correlation with intromission frequency ($-.43 \pm .13$). In other words, males that were slow to achieve their first intromission tended to effect fewer complete copulations in a time-limited test than did those individuals that initiated sexual contact with less delay.

In order to normalize their distribution the latency scores were translated into log values and the result is shown in figure 3. Little

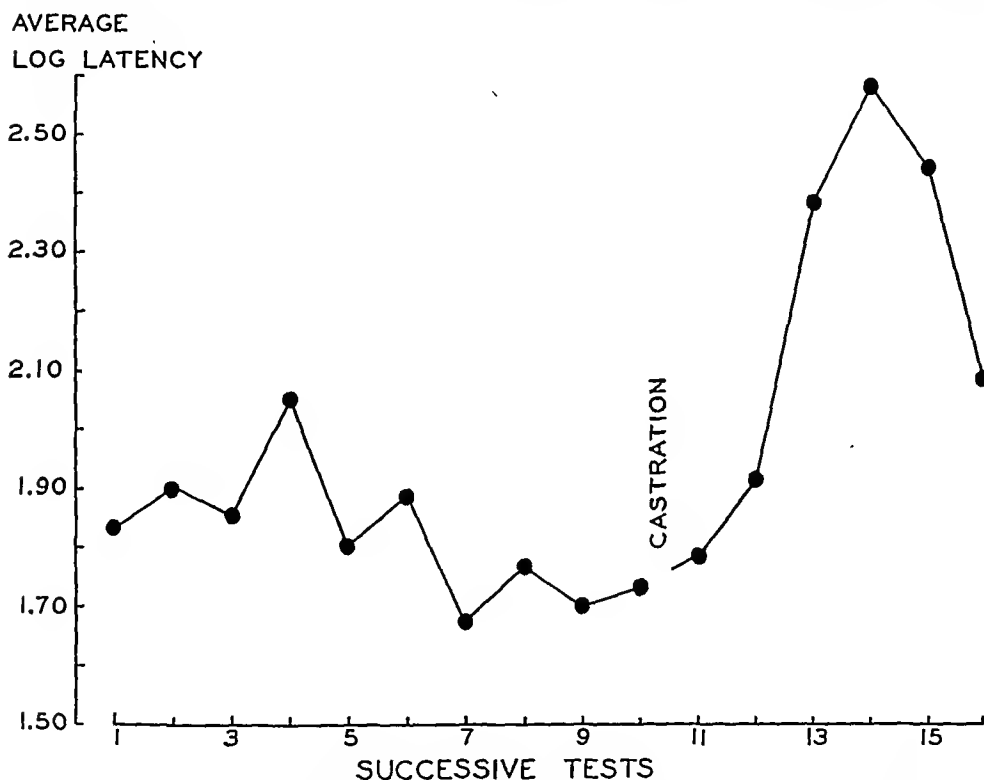


FIG. 3. Average delay before the first intromission in pre- and post-operative tests expressed in terms of log seconds.

change occurred until the third postoperative week, but at this time the delays grew appreciably longer than they had been prior to testicular removal. During the last three tests in the postoperative period so few males achieved intromission that group averages were highly variable. For this reason the apparent decrease in latency on tests 15 and 16 is of doubtful significance but there is little question that the untreated castrate tends to delay for more than the normal period before achieving complete sexual contact with a receptive female.

Effects of androgen treatment

It has been stated that daily injections of testosterone propionate were begun 90 days after castration. Those males receiving 1 micro-

gram per day showed no increase in sexual activity after two weeks of such treatment and accordingly the dosage was increased to 100 micrograms. The remaining animals received 75 micrograms per day from the start of the injection period. Both of these larger doses proved effective in reviving copulatory ability.

Unfortunately it was impossible to continue the experiment until all males had been restored to a normal level of sexuality. However, in five cases this result was achieved after a total of 975 to 1500 micrograms had been administered. In each of these five cases the copulatory performance as measured by intromission frequency equalled or exceeded the average preoperative performance.

Several other individuals displayed increased sexual ability under the influence of testosterone propionate but our results do not permit us to state whether continued treatment would eventually have restored fully normal mating behavior in all cases. The important point which can be made is that in the castrated hamster as in the rat, guinea pig and rabbit exogenous male hormone facilitates the maintenance or restoration of capacity for sexual arousal and for copulatory performance.

DISCUSSION

Although they showed some decrease in weight after castration, our animals appeared to be in excellent health and regained most of the lost weight before the end of the experiment. We do not believe that the weight loss exerted any appreciable effect upon sexual performance. Any effect of operative shock should have been most evident in tests conducted shortly after castration, but in these tests the hamsters showed more intromissions than were executed in subsequent observation periods. Furthermore, as body weight gradually increased there was no parallel increase in sexual activity which is what might have been anticipated if postoperative changes were due primarily to general ill health. Finally, when the administration of testosterone propionate restored mating behavior it did not cause any appreciable change in body weight. It seems safe to conclude that postcastrational changes in sexual performance were due to a deficiency in gonadal hormone and not to some more general systemic effect of the operation.

The effects of castration upon sexual behavior in the hamster and the rat are closely comparable in some respects. If the behavioral change is expressed in terms of the proportion of the group achieving intromission in successive postoperative tests, the performance curves for the two species are practically identical. When intromission does occur after castration the average frequency with which it is achieved in a time-limited test is progressively reduced as the postoperative interval increases. The rate of reduction in this function appears to be about the same in hamsters and rats. Furthermore both species seem to retain some slight capacity for intromission for at least several

months after castration. Finally, sexual behavior can be revived in castrated rats and hamsters by administration of testosterone propionate.

Certain inter-species differences appear to be significant. In the case of the castrated rat abortive attempts to copulate survive somewhat longer than does the capacity for intromission, but these incomplete mating reactions are greatly reduced within a month after gonadectomy. In the hamster, in contrast, this type of sexual response is less easily affected by castration. During the first postoperative month there is little or no decrease in the frequency with which copulation is attempted, and three months after gonadectomy the behavior was still present in 75 per cent of the males examined in the present study. The fact that abortive copulations continue long after the power of intromission has almost disappeared gives a clue to the degree to which these two phases of sexual activity depend upon testicular hormone.

It has previously been shown that in the male rat the ability to achieve intromission may be retained under the influence of hormonal concentrations too low to maintain the power of ejaculation (Beach and Holz-Tucker 1949). In this species, however, it has proven difficult to maintain or restore the incomplete copulatory response without at the same time evoking complete copulation with intromission. In the hamster this separation is readily achieved.

SUMMARY

Nineteen male hamsters were observed in a series of 10 mating tests with receptive females and then castrated. Sex tests were continued during the first six weeks after the operation. Three months postoperatively daily injections of testosterone propionate were initiated and mating tests were resumed.

Castration was followed within two weeks by a marked decrease in the number of males achieving intromission. Within one month this response had been so greatly reduced that it was shown by only one or two individuals in any given testing period. It did not, however, completely disappear from the repertoire of all animals in the space of three months after castration.

The average frequency of intromissions per male per test was definitely reduced within two weeks after operation. Further decreases followed with the result that when this reaction did appear it was executed only two or three times in 15 minutes as contrasted with 40 or more times during similar tests before operation.

Abortive copulatory attempts which did not include intromission occurred in the behavior of all males both before and after castration. The number of tests in which this response appeared was only slightly decreased by castration, and the frequency per male per test was as

high during the six weeks of postoperative testing as it had been before operation.

The amount of time elapsing before a male achieved the first intromission of a given test tended to increase in castrated animals during the first three weeks after gonadectomy. In later tests intromission occurred so infrequently that group averages became highly variable.

Normal mating behavior was revived in 5 of the 19 castrates by androgen treatment which began three months after operation. In other individuals an improvement in sexual performance was noted but injections were terminated before full normality had been restored.

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ANAEROBIC GLYCOLYSIS IN RAT OVARIAN TISSUES DURING PREGNANCY AND LACTATION¹

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SEVERAL reports have been made from this laboratory on the changes in the activities of certain enzymes in ovarian tissues of the rat during different phases of the reproductive cycle. The adenosine triphosphatase activity of corpora lutea and the remaining ovarian tissues has been studied (Biddulph, Meyer and McShan, 1946a, 1946b) as well as the succinic dehydrogenase (Meyer, McShan and Erway, 1945; Meyer, Soukup, McShan and Biddulph, 1947), malic dehydrogenase (McShan, Erway and Meyer, 1948) and the acid and alkaline phosphatases (Stafford, McShan and Meyer, 1947).

Recent advances in the manometric determination of anaerobic glycolysis of tissue homogenates (Racker and Krimsky, 1945; Utter, Reiner and Wood, 1945; and Novikoff, Potter and LePage, 1948) have made it feasible to determine the anaerobic glycolysis of ovarian tissues during pregnancy and lactation in the rat. The method used was essentially that of the latter workers.

MATERIALS AND METHODS

The rats used in this study were of the Holtzman strain, approximately four months of age. The females were bred and the day following insemination was considered to be the first day of pregnancy. The day following parturition was designated the first day of lactation.

The number of young was limited to 6 to 9 until the 5th day of lactation and 6 young thereafter. The animals were killed by decapitation on days 4, 7, 11, 15 and 20 of pregnancy and of lactation, and on the second day of diestrus.

The ovaries were removed immediately and the corpora were dissected out as rapidly as possible on moist hard surface filter paper. During lactation the corpora of pregnancy are distinguished from those of lactation since the latter are lighter in color and have more prominent ovulatory papillae; the corpora of lactation are also larger after day 7 of lactation than those of

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pregnancy. The corpora of each type were counted, weighed collectively and placed in a homogenizing tube containing 0.2 ml. of glass distilled water and which was surrounded by an ice bath. The ovarian residues remaining after dissection of the corpora lutea were weighed and placed in a similar homogenizing tube. The tubes containing the tissues were kept in an ice bath during homogenization and until the homogenates were added to the Warburg flasks. Sufficient cold glass distilled water was added during homogenization to make a 5 per cent homogenate and 0.3 ml. of each kind of homogenate was used in each Warburg flask.

The tissue from two rats was pooled for each determination of diestrous corpora. Sufficient tissue was obtained from a single rat for each determination of the tissues of pregnancy and lactation. If enough tissue was available, duplicate flasks were used. When tissue from a single animal was insufficient for duplicate flasks, only one flask was used. The activity of liver homogenate was simultaneously measured using duplicate flasks so as to show that the purity of the reagents and manipulative procedures were satisfactory.

The method of Novikoff, Potter and LePage (1948) was used for determining the anaerobic glycolysis activity of the ovarian tissues. Slight modifications of this method were found necessary for optimal activity of the tissues used in the present study. Warburg flasks with side arms were used. The flasks contained the following components added in the order cited: Glass distilled water to make a final volume of 3.0 ml., 0.3 ml. of 0.1 M K_2HPO_4 - KH_2PO_4 (pH 7.6), 0.3 ml. of 0.5 M $KHCO_3$, 0.1 ml. of 0.01 M potassium adenosinetriphosphate (ATP), 0.15 ml. of 0.2 M nicotinamide, 0.2 ml. of $\frac{1}{2}$ per cent potassium diphosphopyridine nucleotide (DPN) (added to the side arm) 0.6 ml. of 0.04 M potassium hexosediphosphate, 0.3 ml. of 0.28 M glucose, 0.1 ml. of 0.1 M $MgCl_2$, 0.3 ml. of 0.01 M potassium glutathione (GSH) (when used), 0.2 ml. of 0.015 M potassium pyruvate (freshly prepared from 1.0 M pyruvic acid) and 0.3 ml. of 5 per cent tissue homogenate (equivalent to 15 mg. of fresh tissue). All components except the last three were added before the animal was killed and the flasks were kept at refrigerator temperature until ready for use.

The DPN was prepared according to the LePage (1947) modification of the Williamson and Green (1940) method. Assay by means of the malic dehydrogenase system showed the product to be at least 50 per cent pure. The ATP was prepared as the barium salt according to the method of Necdham (1942). The inorganic phosphorus liberated by boiling ATP in 1 N HCl for 7 minutes showed this product to be 90 per cent pure. Hexosediphosphate was prepared from the barium salt manufactured by Schwarz Laboratories, Inc. Pyruvic acid obtained from the Eastman Kodak Co. was redistilled under vacuum, diluted to approximately 1.0 M and accurately titrated, then diluted and neutralized with K_2CO_3 shortly before use.

After the addition of the homogenate, the flasks were attached to the manometers and gassed from a manifold with 95% N_2 :5% CO_2 for 10 minutes. They were then placed in the bath and equilibrated for 5 minutes. The DPN was then tipped in from the side arms. After 5 minutes the zero reading was taken and the measurement of CO_2 evolution begun. Readings were taken at 10 minute intervals for the next 80 minutes. It was found that the rate of reaction was consistently linear with respect to time from minutes

20 to 80; for this reason the $Q_{CO_2}^{N_2}$ values (microliters of CO_2 given off under anaerobic conditions per mg. of dry tissue per hour) were based on this period. The dry weights used for the calculations of this Q value were those obtained by Meyer et al. (1947) (Diestrous corpora, 24.4%, corpora of pregnancy, 22.8%, corpora of lactation, 21.4% and ovarian residue, 22.4%).

RESULTS AND DISCUSSION

The anaerobic glycolytic activity of the ovarian residue throughout pregnancy and lactation and of the corpora of diestrus, pregnancy and lactation, together with their weights are recorded in Table 1.

TABLE 1. GLYCOLYTIC ACTIVITY OF LUTEIN TISSUE AND OVARIAN RESIDUE AND WEIGHT OF CORPUS LUTEUM DURING PREGNANCY AND LACTATION

Stage	No. of expts.	Glycolytic activity ($Q_{CO_2}^{N_2}$) Corpora of:		Ovarian residue	Weight (mg.) Corpora of:	
		Pregnancy	Lactation		Pregnancy	Lactation
Diestrus	6	$10.2 \pm 2.54^*$		—	$1.22 \pm 0.14^{**}$	
Pregnancy days						
4	4	16.4 ± 1.84		10.2 ± 1.07	1.60 ± 0.17	
7	4	16.8 ± 0.99		9.6 ± 4.17	1.78 ± 0.15	
11	6	17.4 ± 1.79		9.3 ± 3.29	2.50 ± 0.62	
15	6	16.0 ± 1.43		7.5 ± 1.85	4.23 ± 0.28	
20	4	16.0 ± 1.82		8.8 ± 1.91	4.40 ± 0.15	
Lactation days						
4	5	12.8 ± 0.70	16.6 ± 1.69	5.2 ± 0.68	2.65 ± 0.16	1.83 ± 0.19
7	2	14.4	18.2	6.1	1.84	2.18
11	4	14.0 ± 2.33	18.1 ± 1.18	5.8 ± 3.67	1.42 ± 0.12	2.11 ± 0.11
15	4	—	17.2 ± 0.71	5.5 ± 1.68	—	2.56 ± 0.16
20	4	—	17.5 ± 0.94	7.2 ± 4.36	—	2.38 ± 0.33

* Standard deviation.

** This figure is not a true standard deviation. The desired corpora from each rat were weighed collectively and the average corpora weight calculated from the total weight. This figure is the standard deviation from the average weight determined in each collective weighing.

The glycolytic activity of the corpora together with their weights are graphically presented in Figure 1. The weights of the corpora of pregnancy and of lactation are essentially the same as those obtained by Biddulph, Meyer and McShan (1946a).

There is a marked increase in the glycolytic activity of the corpora from the second day of diestrus to the 4th day of gestation. From day 4 to 11 of pregnancy there is no significant change in activity. After day 11 there is a slight decline in activity of the corpora of pregnancy until day 20, and a more rapid decrease to the 4th day of lactation. Statistical analysis of the data on hand shows only two significant changes in the glycolytic activity of lutein tissue. The first of these is between the second day of diestrus and day 4 of preg-

nancy while the second such change occurs in the corpora of pregnancy between day 20 of pregnancy and day 4 of lactation.

The enzymatic activity of the diestrous corpora was determined in order that it might serve as a base line with which to compare the enzymatic activity of lutein tissue during pregnancy and lactation. It can not be assumed, however, that the corpora of diestrus are functionally similar to those of early pregnancy, since it is believed by some workers that the corpora of diestrus are non-functional, or, at most, under low-grade stimulation (Everett, 1945), while those of early pregnancy are highly functional and under the direct stimula-

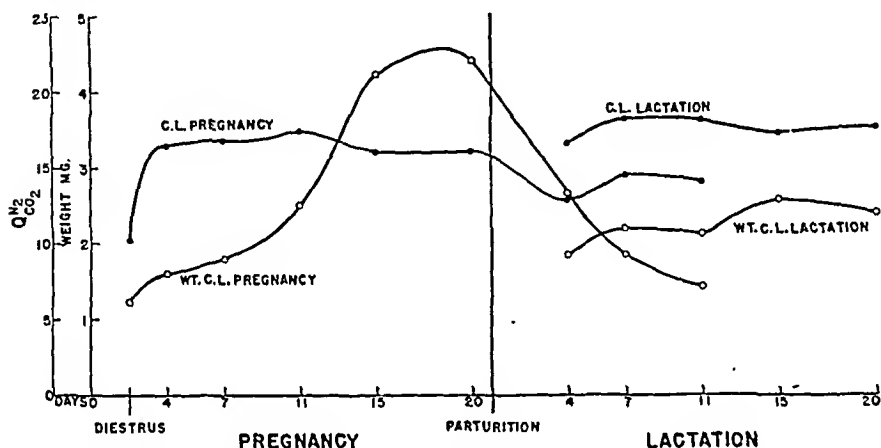


FIG. 1. The anaerobic glycolysis and weight of the corpora lutea of pregnancy during pregnancy and lactation, and of the corpora of post-partum ovulation during lactation.

tion of the pituitary (Everett, 1947). This difference in the functional state could well be reflected in the differences in the glycolytic activity of the two types of corpora.

There is no appreciable change in the activity of the corpora of post-partum ovulation except that it is higher than the activity of the corpora of pregnancy. The glycolytic activity of the ovarian residue shows no noteworthy change other than a gradual decrease during pregnancy and lactation.

In order to check the likelihood that the reconstituted system used in these determinations might be limiting the enzymatic activity, homogenates of other rat tissues such as kidney, brain and liver were tested under identical conditions. The glycolytic activity of these tissues was substantially higher than that of luteal tissue. The activity of liver in terms of $Q_{CO_2}^{Ni}$ was as great as 42. From these data it is evident that the system used will measure greater enzyme activity than that present in the ovarian tissues.

It has been found in this laboratory (unpublished data) that glu-

tathione will activate the anaerobic glycolysis of liver and brain homogenates as measured by lactic acid production and CO_2 evolution. The mechanism of this activation is, at present, not clear but is probably concerned with the protection of the $-\text{SH}$ enzymes of the glycolytic system (see Barron and Singer, 1943; and Cori, Slein and Cori, 1948). It should be noted, however, that LePage (personal communication) found that GSH had no effect on the glycolytic rate although his procedure did not differ from ours in any discernible manner.

Whatever the final outcome of this activation study may be, when this finding was applied to luteal tissues, it was found that the activity of diestrous corpora was increased approximately 45 per cent. By

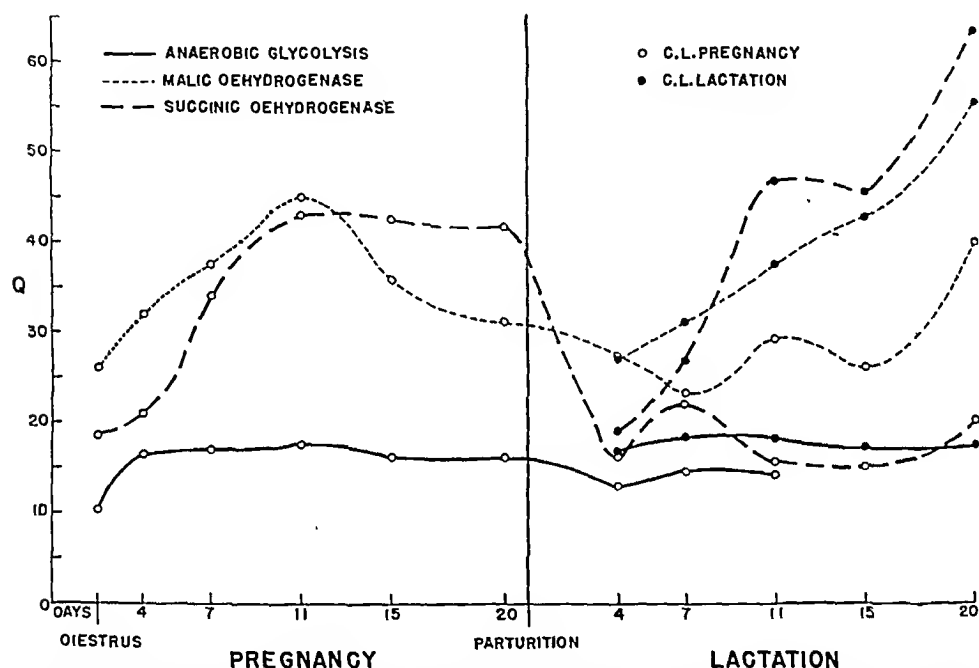


FIG. 2. The activities of the three enzyme systems, anaerobic glycolysis (Q_{GL}^N), malic dehydrogenase (Q_{MD}) and succinic dehydrogenase (Q_{SD}), of the corpora lutea of pregnancy and of the corpora lutea of post-partum ovulation.

contrast, the glycolytic activity of luteal tissues from various stages of pregnancy and lactation was activated 5 to 25 per cent by the addition of glutathione. With the addition of this tripeptide the variations of the glycolytic activity of the lutein tissue depicted in Figure 1 tend to be abolished and there is no significant change in the activity during the stages studied. If the *in vivo* glycolytic rate of luteal tissue is more accurately represented by the measured *in vitro* GSH-activated rate, then there is no essential change in the glycolytic activity during the formation, growth and regression of the corpus luteum.

It is of interest to compare the changes in activity of this anaerobic system in luteal tissue with the changes of the oxidative enzymes of carbohydrate metabolism which have been studied previously in this

laboratory (Meyer et al., 1947 and McShan et al., 1948). With the corpora of pregnancy the succinic dehydrogenase (SDH-ase) activity increases very little from diestrus to day 4 of pregnancy. From day 4 to day 11 the SDH-ase activity rises markedly to its peak and remains at that level until day 20 of pregnancy after which there is a rapid and maintained decrease. The SDH-ase activity of the corpora of lactation shows an over-all continuous increase from the 4th day of lactation to day 20. The malic dehydrogenase (MDH-ase) activity of the corpora of pregnancy shows a regular increase from diestrus to day 11. This activity is not maintained but falls off, reaching a low point at day 7 of lactation and rising to a high value again on the 20th day of lactation. The MDH-ase activity in the corpora of lactation increases regularly from day 4 to day 20. In contrast to these pronounced changes in activity of the enzymes of the aerobic oxidative cycle including cytochrome oxidase (McShan, Erway and Meyer, 1948), the anaerobic glycolytic activity of luteal tissue appears to be quite constant throughout pregnancy and lactation (see Figure 2).

There is some similarity between the changes in glycolysis and adenosinetriphosphatase (Biddulph, Meyer and McShan, 1946a) for the ATP-ase of the corpora of pregnancy does not change until shortly before parturition after which there is a marked increase in concentration. There is also no marked change in the ATP-ase of the corpora of lactation until day 20.

Consideration of the marked changes in the dehydrogenase activities in contrast to the stability of the glycolytic activity raises the question of substrate turnover of these complementary systems since one of the main pathways of pyruvate metabolism is through the Krebs tricarboxylic acid cycle. If the Q values for these three enzyme systems are recalculated in terms of micromoles of lactate (or pyruvate) produced and succinate and malate used, striking differences in the comparative activities of these enzyme systems are seen. These data are summarized in Table 2. It is worth noting that, in these systems, the two dehydrogenases have the ability to act on two to seven times as much substrate as that produced by the glycolytic enzymes.

That the amount of substrate produced by glycolysis *in vivo* might be even less than that calculated in Table 2 is indicated by studies of the Pasteur effect on luteal tissue. Determination of this effect showed approximately 30 per cent inhibition of glycolysis by oxygen (i.e., for corpora of day 20 of pregnancy: $Q_{Co}^{N_2} = 16.1$, $Q_{Co}^{O_2} = 11.7$). This finding would indicate that glycolysis may be less *in vivo* than that measured under anaerobic conditions and that the glycolytic scheme is actually contributing relatively less substrate to the Krebs cycle than that calculated in Table 2.

Since the substrate for the Krebs cycle in the scheme of carbohydrate metabolism is pyruvate derived from the activity of the gly-

colytic enzymes, it is tempting, on the basis of the above discussion, to postulate that the relatively high activity of the dehydrogenases in the luteal tissue indicates an extra-carbohydrate source of substrate for the Krebs cycle. Such a source is well known for tissues such as liver and kidney, but has not been demonstrated for ovarian tissues.

If the same relationship between the activity of the glycolytic and oxidative enzymes which is found *in vitro* holds true *in vivo* then it follows that compounds not produced by glycolysis in the lutein tissue are made available for the tricarboxylic acid cycle. It is possible

TABLE 2. THE RATES OF LACTATE PRODUCTION, SUCCINATE AND MALATE UTILIZATION BY LUTEAL TISSUE*

Stage	Micromoles of lactate produced		Micromoles of succinate used		Micromoles of malate used	
	Corpora of: Preg.	Lact.	Corpora of: Preg.	Lact.	Corpora of: Preg.	Lact.
Diestrus	0.46		1.65		2.32	
Pregnancy days						
4	0.73		1.86		2.85	
7	0.75		3.02		3.34	
11	0.78		3.82		4.00	
15	0.71		3.78		3.19	
20	0.71		3.70		2.77	
Lactation days						
4	0.57	0.74	1.43	1.68	2.43	2.40
7	0.64	0.81	1.95	2.38	2.06	2.77
11	0.63	0.81	1.38	4.16	2.62	3.34
15		0.77	1.34	4.04	2.32	3.80
20		0.78	1.80	5.65	3.58	4.94

* Recalculated from the Q values in terms of micromoles per mg. dry tissue per hour.

that lactate of the blood is used by this tissue. However, it is more likely that the lutein tissue extracts such substances as amino acids and fatty acids from the blood as additional substrates for the Krebs cycle. It has been reported (Talbert, Stafford, Meyer and McShan, 1948) that there is a definite increase in the vascular pattern of the corpora during the latter part of pregnancy which correlates roughly with the increase in SDH-ase, MDH-ase and cytochrome oxidase activities.

In reference to the transport of extra-cellular substances into the lutein cells it should be pointed out that alkaline phosphatase of this tissue increases in late pregnancy (Stafford et al., 1947) and that in lutein tissue this enzyme is confined largely to the vicinity of the endothelium (Talbert et al., 1948).

The possibility also exists that the additional substrates for the tricarboxylic acid cycle may be derived in part from intra-cellular sources. Laqueur and Koets (1945) have shown that the total lipid content of the corpora lutea of pregnancy decreases from diestrus to day 16 of pregnancy and that there is a sharp rise in these lipids about the time of parturition. The protein content of the corpora follows the same general pattern. With reference to these changes it is noteworthy that the activities of both SDH-ase and MDH-ase are high in the latter part of pregnancy and drop off markedly at the time of parturition.

These additional data, when correlated with the previous discussion, provide a further basis for postulating that lutein tissue has the ability to metabolize fats and proteins.

There is an abundance of data available for comparing the changes in the activities of various enzymes during carcinogenesis. These studies have shown that regardless of the variable enzyme patterns of the parent tissues, the tumors arising from these tissues are characterized by a similar enzyme pattern. The present authors, however, are not in a position to make a comparison of the genesis and growth of luteal tissue to the genesis and growth of tumor tissue, since there are no data available concerning the enzyme systems in the follicular cell from which the lutein cell develops.

On the other hand, a justifiable comparison of enzyme pattern can be made between the rapidly growing luteal tissue and malignant tumors. As noted in the previous discussion, the potential glycolytic activity of luteal tissue during the phase of rapid growth is low while the activities of succinic dehydrogenase and malic dehydrogenase are relatively high during the same period. By contrast, a malignant tumor such as the Flexner-Jobling carcinoma possesses an entirely different type of enzymatic pattern. It has been shown (Novikoff et al., 1948 and LePage, 1948) that this tissue has a high glycolytic activity while enzymatic reactions characteristic of the Krebs tricarboxylic acid cycle, such as succinic dehydrogenase (Schneider and Potter, 1943) and oxalacetate oxidation (Potter, Pardee and Lyle, 1948), are low or negligible. It has been further shown that tumor homogenates do not have the ability to oxidize significant amounts of oxalacetate, even in the presence of a maintained high energy phosphate reservoir (Potter and LePage, 1949). Thus, it is evident that luteal tissue and tumors differ markedly in the enzymatic pattern concerned with oxidative metabolism even though they are both rapidly growing tissues.

SUMMARY

The anaerobic glycolysis of the lutein tissue and of the remaining ovarian tissue was determined during diestrus, pregnancy and lactation in the rat.

The glycolytic activity of the corpora of pregnancy increases sharply from the second day of diestrus to the 4th day of pregnancy and remains essentially unchanged throughout pregnancy. There is a significant drop in the activity of this tissue from day 20 of pregnancy to the 4th day of lactation. In the corpora of lactation, there is no significant change in activity.

The changes in the glycolytic activity of the lutein tissue are discussed in relation to the previously determined changes in succinic and malic dehydrogenases of this tissue.

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ON THE INFLUENCE OF 3-KETO-STEROIDS ON THE PHOSPHATASE CONTENT OF ATYPICALLY PROLIFERATING UTERINE EPITHELIUM IN THE GUINEA PIG

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INTRODUCTION

THE REMARKABLE findings of Huggins and his students (1941, 1943) with the increase of acid phosphatase in the blood serum in cases of prostatic cancer, and its decrease after castration or administration of estrogen, are amply known. The work of Huggins was preceded by that of Gutman and Gutman (1938) who were the first to report that development of prostatic epithelia dependent on, or induced by, testosterone was accompanied by an increase of acid phosphatase. These statements have been recently substantiated by new work of Huggins on the prostate under different experimental conditions (Pazos and Huggins, 1945; Huggins and Russel, 1946).

The question arose whether this specific action of androgen on the cellular enzyme pattern was exclusive of this group of steroids, or whether it was shared by all those steroids which are able to induce cellular proliferation. There was likewise the question whether the decrease of prostatic acid phosphatase as induced by estrogens was exclusive of this steroid, or whether this action was shared by other steroids able to counteract steroid-induced growth. If the above concept was true the phosphatase content should increase in estrogen-induced atypical uterine growth, and this increase should be antagonized by antiestrogenic 3-ketosteroids whose antitumorigenic actions have been under study for a long time (see summary Lipschutz et al., 1948).

As early as 1942 a project was elaborated, in association with Prof. Lipschutz, to study the phosphatase content in estrogen-induced epithelial and conjunctive growth and likewise the influence antiestrogenic 3-ketosteroids may have on the phosphatase content in similar growth. In preliminary work of Fuenzalida and Sarras² the

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TABLE 1*

Estrogen used	Absorbed per day μ g.	Duration of exper. days	Weight of uterus grams	Number of animals	Phosphatase units (average)				No. of animals with alkal. phosphat. above upper limit			
					Mucosa		Myometrium†					
					Mucosa		Acid		Alkal.	Acid	Mucosa	Myo- metrium
					Alkal.	Acid						
0	0	0		28	54†	27§	31	6.3	0	0		
benzestrol diprop.	2 to 4 (injections)	37-101 (73)	1.8-19.0 (5.8)	37	119	18	56	5	23	5		
diethylstilbestrol	280 (subcut. tabl.)	63- 90 (73)	2.1- 9.8 (4.3)	31	80		62	—	12	5		

* Comprises experiments made in collaboration with Drs. M. Pinto, R. Nahmías, A. Santa-María, C. Díaz and J. Tenorio, working for their M.D., Universidad de Chile, 1946 to 1948.

† Or entire uteri; 28 animals with the determination of alkaline phosphatase; 23 animals with the determination of acid phosphatase.

‡ 12 animals.

§ 7 animals.

following results were obtained: (1) there was a considerable increase of the alkaline phosphatase content of the uterus in guinea pigs to which different estrogens were administered for several months and in which an increase of the uterine weight had taken place; (2) there was no increase of acid phosphatase in the uterus; (3) there was no increase of alkaline or acid phosphatase in estrogen-induced uterine or extrauterine abdominal fibroids; (4) when experiments were prolonged for 3 months or more and excessive uterine weights of 12 g—instead of 1 g in the normal animal—were obtained, the alkaline phosphatase content began again to decrease. On the basis of the statements (1) and (3) the following two tentative suggestions were made to explain the decrease in experiments of long duration: (a) that there was no notable increase of alkaline phosphatase in the growing myometrium as there was none in fibroids, and that the increase of uterine alkaline phosphatase was due predominantly to the proliferating uterine epithelium, in such a way that with the considerable growth of the myometrium the alkaline phosphatase content of the epithelium *seemingly* again decreased; (b) that the alkaline phosphatase content of the uterine epithelium *really* decreased under the influence of the epitheliotoxic action of estrogen so often observed in similar experiments. To prove these suggestions, phosphatases were determined in all subsequent work separately in the myometrium and in the epithelium removed, together with the submucosa, from the myometrium.

METHODS

The uterus was weighed and opened by scissor or knife. The epithelium together with the submucosa (to simplify: "mucosa") was separated by scraping with a blunt scalpel. The myometrium and mucosa were weighed separately. The myometrium was triturated; distilled water was added so as to obtain a concentration of 1 mgm of myometrium, or of mucosa, per 1 ml. The material was kept at 4°C for 24 hours. The method of King and Armstrong (King et al., 1942) was used for the determination of phosphatase (disodium-phenyl-phosphate as substrate). Readings were made with Klett's photocolormeter with filter 66. All data refer in the following to King-Armstrong units per gram of fresh tissue. In our former work with Sarras, the method of Kay (Kay, 1930) was used and for this reason these results are omitted in the present paper.

Influence of estrogen on the phosphatase content of myometrium and mucosa

Results with 91 castrated guinea pigs are given in table 1.

In our attempts to separate the myometrium and mucosa of normal uteri we often failed; there were only 12 animals with the separated determination. Results with the entire uterus and the myometrium were pooled together. The highest values obtained with the separate determination in the mucosa and myometrium was taken as

TABLE 2

Stilbestrol absorbed per day μg.	3-ketosteroids		Duration of exper. days	Weight of uterus gr.	No. of animals	Mucosa		Myometrium		No. of animals with alkal. phosphat. above normal upper limit	
	used	absorb. per day				Alkal. phosph. un.	Acid phosph. un.	Alkal. phosph. un.	Acid phosph. un.	Mucosa	Myo- metrium
280	0	0	63-90 (73)	2.1-9.8 (4.3)	31	80	—	62	—	12	5
214	Progester.*	213	30-150 (92)	1.0-2.8 (1.7)	23	39	26	39	9	2	2
245	Progester.†	45	54-93 (77)	1.0-4.6 (2.44)	30	32	—	49	—	0	3
296	Testost. propion.	265	61-63 (62)	2.8-9.9 (4.3)	6	26	—	37	—	0	0
237	Dihydrotestost.	82	35-153 (86)	0.6-5.2 (3.0)	21	49	46	63	13	2	3
190	Desoxycorticost.	198	30-124 (83)	1.5-8.8 (3.0)	21	107	21	36	6	9	1

* Tablets of pure progesterone.

† Tablets containing but 40 % of progesterone.

the normal upper limit which was for alkaline phosphatase of the mucosa 87 and of the myometrium 89.

Benzestrol dipropionate and diethylstilbestrol were used as estrogens. The first was administered by subcutaneous injection of oily solutions; injections of 5 and 10 μ g of benzestrol were given thrice weekly. Stilbestrol was absorbed from subcutaneously implanted tablets containing 90% of stilbestrol and 10% of cholesterol. The animals were killed after a treatment of 1 to 3 months. With benzestrol the alkaline phosphatase content of the mucosa surpassed the upper limit of normal uteri in no less than 23 out of 37 cases; with stilbestrol in 12 out of 31 cases. There was no increase of acid phosphatase in the mucosa. There were only 5 out of 37 animals and 5 out of 31 animals with an increase of alkaline phosphatase in the myometrium. Determination of acid phosphatase in the mucosa and of both phosphatases in the myometrium were regularly made in subsequent work without an increase of acid phosphatase and rarely an increase of alkaline phosphatase being noted; consequently the respective data will be omitted here.

We have referred in the Introduction to the decrease of uterine alkaline phosphatase in experiments with the prolonged action of estrogen. Results with benzestrol (fig. 1) show clearly that there was a gradual decrease even when the mucosa was examined separately; the number of animals beneath the normal upper limit was much greater at 3 months than at 40 to 50 days. Less evident were the results with stilbestrol (fig. 2) probably due to not disposing of experiments of 40 to 50 days for comparison. The results speak in favor of the concept that with the more prolonged action of estrogen the alkaline phosphatase shows a tendency to diminish again. Indeed, the objection can be made that in experiments of longer duration there was a greater development of loose or oedematous conjunctive tissue and this may have stultified the results.

Influence of antiestrogenic steroids on the estrogen-induced increase of the alkaline phosphatase content

Results with 101 animals are summarized in table 2.

In all experiments stilbestrol absorbed from subcutaneously implanted tablets was used. The antiestrogenic steroids also were absorbed from similar tablets. In some cases tablets containing only 40% of the specific steroid were used.

With *progesterone* the antiestrogenic actions as described in former work of Lipschutz and his students became evident: closure of the vagina, decrease of uterine weight, prevention of fibroids, but no inhibition of mammary growth. Only in 2 out of 53 animals the normal upper limit has been surpassed (fig. 3). Similar results were obtained with *testosterone propionate* and *dihydrotestosterone*: the normal upper limit has been surpassed in only 2 out of 27 animals.

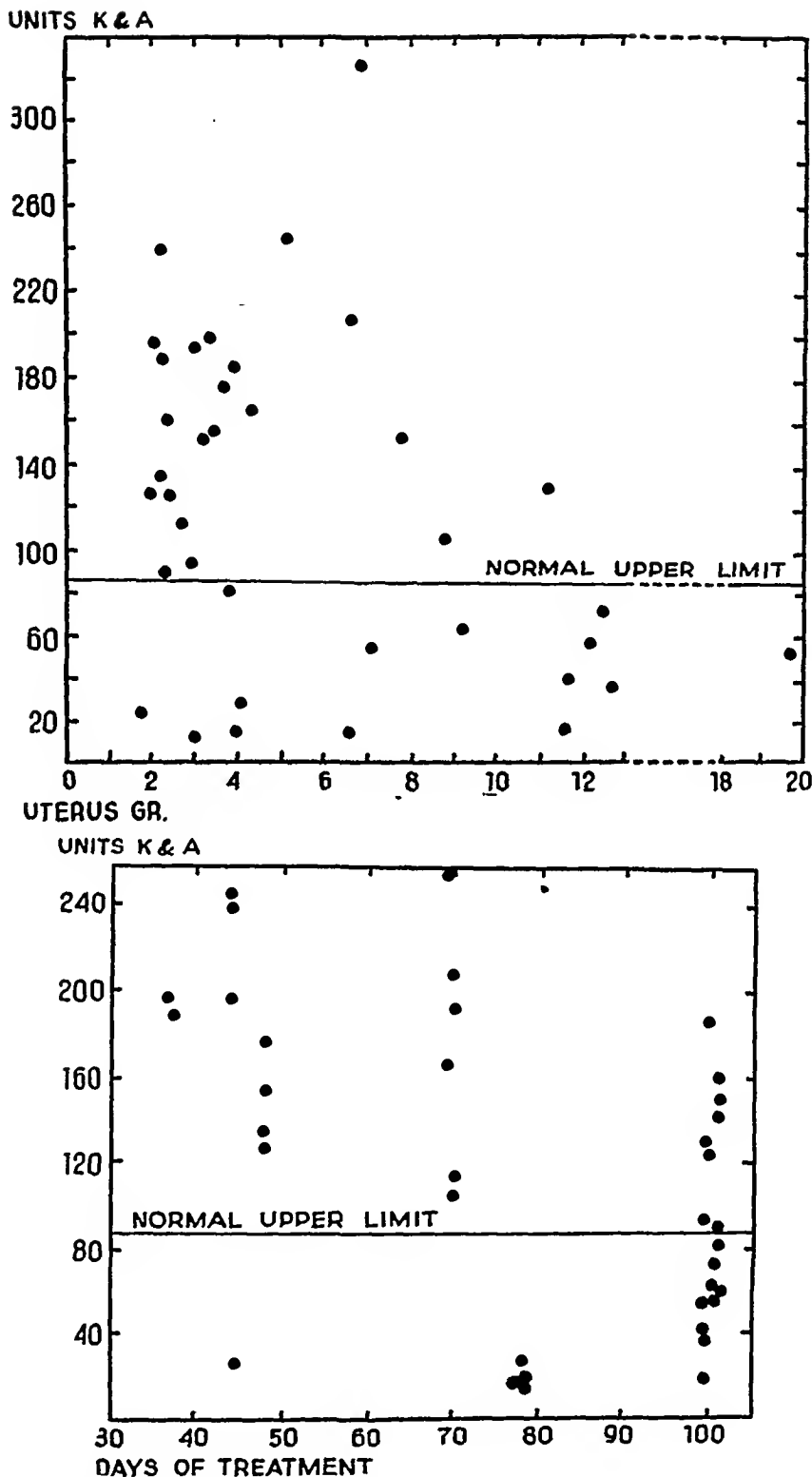


FIG. 1. Alkaline phosphatase in uterine mucosa (or submucosa) of guinea pigs treated with benzzestrol dipropionate. King & Armstrong units per gram.—A. The greatest frequency of increase is in uteri of 2 to 5 g.—B. The greatest frequency of increase is at 37 to 48 days. At 78 days, and specially at 100 days, there is again a considerable number of animals with normal values.

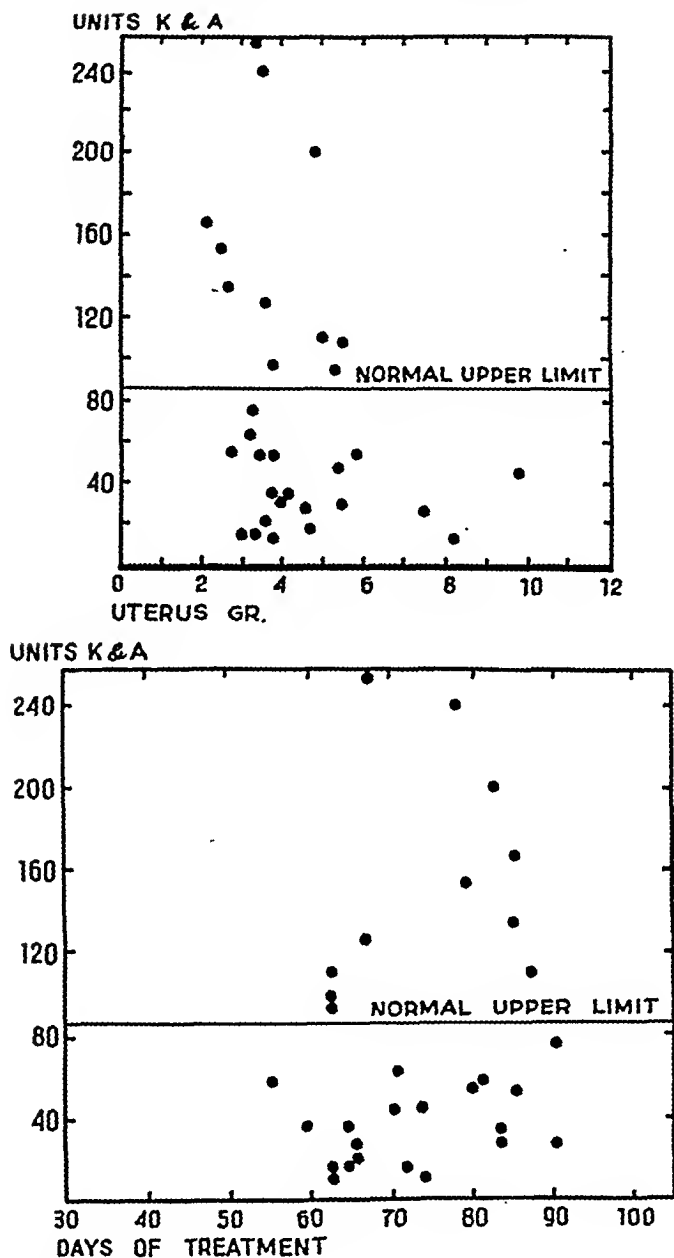


FIG. 2. Alkaline phosphatase in uterine mucosa (or submucosa) in guinea pigs treated with diethylstilbestrol. For details see text.

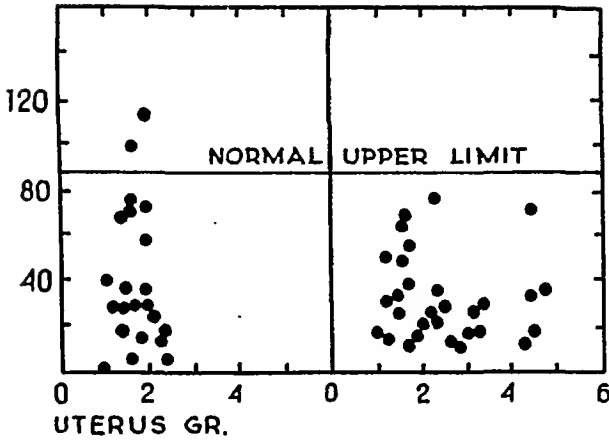


FIG. 3. Influence of progesterone on the alkaline phosphatase content of uterine mucosa (or submucosa) in animals receiving simultaneously stilbestrol. Compare to fig. 2A.

The difference between the stilbestrol group, on the one hand, and the groups with stilbestrol and antiestrogenic steroids on the other hand was striking. The percentage of animals above the normal upper limit with stilbestrol alone was about 40, with progesterone or androgens about 4 to 8. Inhibition of estrogen-induced increase of alkaline phosphatase with androgens was less pronounced than with progesterone (fig. 4). This was concomitant with a less pronounced antifibromatogenic action; there were various animals with fibroids.

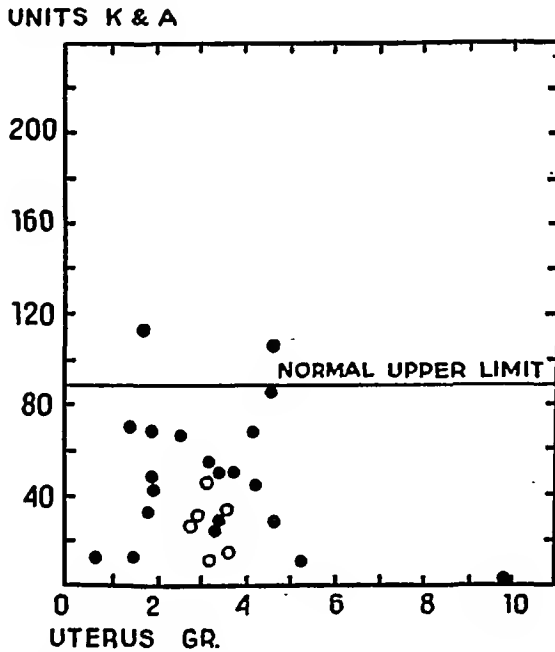


FIG. 4. Influence of androgens on the alkaline phosphatase content of uterine mucosa (or submucosa) in animals receiving simultaneously stilbestrol. O testosterone propionate; ● dihydrotestosterone. Compare to fig. 2A.

Contrary to our expectation no decrease of alkaline phosphatase was obtained with desoxycorticosterone. This was probably due to the quantities absorbed having been insufficient; fibroids were prevented but the vagina remained open.

DISCUSSION

When discussing the quantitative results with the action of different steroids as given in tables 1 and 2 it seems preferable to consider the relative number of animals in which the phosphatase content increased above the normal level, instead of comparing averages. There was in our work, as exemplified by figures 2 and 4, no frequency

TABLE 3

Substances used	No. of animals total (n)	Above normal upper limit (z)	Percentage $\frac{z+1}{n+2} \cdot 100$	σ of the percentages*
Stilbestrol	31	12	39	8.3
Stilbestrol and progesterone	53	2	5	2.9
Stilbestrol and testosterone propionate	6	0	13	11.2
Stilbestrol and dihydrotestosterone	21	2	13	6.8
Stilbestrol and desoxycorticosterone	21	9	43	10.1

$$* \sigma p = \sqrt{\frac{p(100-p)}{n+3}}$$

distribution around a statistical mode but very disperse values. Under these circumstances we must center the discussion around the question whether the differences between the percentages of animals above the normal upper limit are significant.

Percentages should be calculated according to the formula $p = \frac{z+1}{n+2} \cdot 100$, in which p is the corrected percentage, z the number of positive cases and n the total number of cases (Van Der Waerden, 1936). Results are given in table 3.

The difference between the stilbestrol group and the stilbestrol-progesterone group is striking, and so also the difference between the stilbestrol and the stilbestrol-androgen groups. There can be no doubt that progesterone and so also androgens counteract the estrogen-induced increase of alkaline phosphatase in the uterine mucosa. An even more striking picture of this action of the mentioned steroids is obtained when only those experiments are compared in which uterine weights were equal. Such a selective comparison is made in table 4.

Our results may be compared to those which have been obtained in recent years with cytochemical studies of the phosphatase content in the uterus. Atkinson and Elftman (1946, 1947) stated an increase of alkaline phosphatase in the uterus of castrated mice with the administration of estradiol benzoate during three days; there was an increase not only in the endometrium but also in the myometrium and especially in the longitudinal layer. Dempsey et al. (1949) found that alkaline phosphatase disappeared completely in the uterus of the rat 26 days after hypophysectomy and that the phosphatase content was restored to normal when whole pituitary powder was given.

TABLE 4

Group	No. of animals		Percentage above normal upper limit	Significative difference*
	Total	Above normal upper limit		
Stilbestrol	19	7	38	
Stilbestrol and progesterone	53	2	5	3
Stilbestrol	31	11	37	
Stilbestrol and testosterone propionate	6	0	13	2
Stilbestrol	23	8	36	
Stilbestrol and dihydrotestosterone	21	2	13	2

* $D = \frac{p_1 - p_2}{\sqrt{\sigma_{p_1} + \sigma_{p_2}}}$. Values of D equal to or above 2 are considered significant.

Atkinson and Engle (1947) reported an increase of alkaline phosphatase in the endometrium of women and *M. rhesus*; it was remarkable that the increase was limited to the proliferative or estrogenic phase, there being a decrease in the secretory or luteal phase. On the other hand, there was always an increase in the work of Atkinson and Elftman (1946) with estrogen in mice when progesterone or testosterone propionate were added. This is contrary to our results with progesterone. But this might have been due to quantities too small or to a duration too short. It must also be held in mind that in our work we are dealing with atypical proliferation of the endometrium; indeed it does not seem very likely that there should be such a definite difference as to the influence of progesterone in typical and atypical proliferation of the same cells.

All these findings are acquiring gynecological interest as evidenced by the work of Zondek and Hestrin (1947) on the phosphorylase content in the endometrium of primates and women and its bearing to sterility. Arzac and Blanchet (1948) have studied the alkaline phosphatase content and glycogen in endometrial biopsies.

SUMMARY

A considerable increase of chemically determined alkaline phosphatase took place in the uterus of the guinea pig under the influence of a prolonged treatment with estrogen.

This increase was localized mainly in the greatly hypertrophied uterine mucosa, or submucosa.

The alkaline phosphatase content of the uterine mucosa, or submucosa, may become several times that of a non treated animal.

When experiments were prolonged for several months, alkaline phosphatase showed the tendency to decrease again.

When progesterone was administered simultaneously with estrogen the alkaline phosphatase increase in the mucosa, or submucosa, was counteracted so as to attain, under certain quantitative conditions, the same level as in non treated animals.

A similar though less pronounced anti-phosphatase action was observed with androgens as testosterone propionate and dihydrotestosterone. No such action was found with desoxycorticosterone.

There was only slight increase of alkaline phosphatase in the myometrium when greatly hypertrophied under the influence of the prolonged treatment with estrogen.

No increase of acid phosphatase took place in any part of the uterus under the influence of estrogen.

There was no increase of alkaline or acid phosphatase in estrogen-induced uterine or extrauterine abdominal fibroids.

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THE FETAL BOVINE THYROID: MORPHOGENESIS AS RELATED TO IODINE ACCUMULATION¹

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IN THE fetal calf, which has a gestation period of approximately 285 days, thyroxine-like iodine can be detected in the thyroid gland on the sixtieth day of intrauterine life. Thereafter, the amounts of thyroxine-like and total iodine in the gland increase steadily, and the rates of increase of these two iodine fractions have been shown to be related exponentially to fetal body weight, length, and age (Wolff *et al.*, 1949). Since, in addition, the *concentration* of iodine in the developing gland (i.e., mg. of iodine per 100 gm. fresh tissue) appeared to increase progressively, it was postulated that with increasing age, the fetal thyroid acquired an increased capacity for iodine storage.

In the present investigation, we have attempted to correlate the morphogenesis of the gland with its iodine content. Since the follicle might be regarded as the structural and functional unit of the gland, we sought to determine the relation of its development to the capacity of the gland to store iodine.

EXPERIMENTAL

Fifty-nine thyroid glands were collected from fetal calves ranging in age from 53 to 265 days in intrauterine life (gestation 278–285 days). The age of each fetus was determined as described in an earlier report (Nichols *et al.*, 1949). The methods employed for iodine determinations have also been described (Wolff *et al.*, 1949). The glands were fixed in Bouin's fluid, embedded in nitrocellulose, and sectioned at six micra. The sections were stained with hematoxylin and eosin and also by the Mallory-Azan method.

RESULTS

The thyroid glands have been grouped according to stages of histogenesis as shown in Table 1. At the earliest stage (53 to 70 days), the glands consisted of proliferating masses of branching epithelial

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plates or cords, one to three cells wide, surrounded by well developed blood and lymphatic capillaries (Plate I, fig. 1). The interstitial mesenchymal connective tissue was not abundant at that time, except in the center of the lateral lobes or in the isthmus. Many nuclei were undergoing mitosis, indicating rapid growth of the epithelial cords. The individual cells adhered to one another, but were sharply outlined. The abundant, lightly staining cytoplasm of these cells contained no visible colloid droplets.

Colloid droplets (staining with either aniline blue or with orange G) first made their appearance in the cell cytoplasm of thyroid glands in 75- to 88-day-old fetuses. These droplets moved toward the cell

TABLE 1. IODINE CONTENTS OF FETAL THYROIDS

Estimated age range of group	Number of glands examined		Average iodine values mg. per cent			Reference to figures (Plates I-III)
	Histo-logically	Chemically	Thyroxine	Non-thyroxine	Total*	
days						
53-70	7	4	3.0	14.1	17.1 ± 5.5	1
75-88	6	12	4.0	7.3	11.3 ± 1.5	2-6 and 9
90-105	14	14	2.9	9.6	12.7 ± 2.0	10
106-118	6	9	6.3	11.6	17.9 ± 3.1	11
120-133	4	6	5.5	14.5	20.0 ± 4.5	12
135-152	7	11	8.5	24.0	32.6 ± 2.9	13
153-172	5	13	10.8	23.3	34.1 ± 4.8	14
175-205	3	4	19.1	48.0	67.0 ± 7.1	15
218-265	5	8	35.1	82.4	118.0 ± 15.0	16

$$* \text{ Standard error of mean } = \sigma_{\bar{x}} = \sqrt{\frac{\sum_i^n (x_i - \bar{x})^2}{n(n-1)}}$$

border and eventually passed into intercellular spaces marking the center of the future follicle (figs. 3 and 4).

Steps in the development of true follicles are clearly shown in figs. 4, 5, and 6. During the follicle formation, the regularity of cord arrangement became less distinct (fig. 2).

Photomicrographs 9 to 16 (all of the same magnification) show the gradual development of the thyroid parenchyma. In the process of reconstruction into follicles, the mass of epithelial cords (now constituting interfollicular tissue) gradually diminishes and, in the later stages of thyroid development, this interfollicular tissue is represented by rather small islets of cells. At the periphery of the gland, or, in later stages, at the periphery of the lobules, the cord arrangement remains as an uninterrupted rim up to 150th day of intrauterine life. This tissue apparently serves as a reserve material for the growing thyroid since numerous mitoses and very young follicles are present.

Intracellular colloid formation and growth of the follicles were not appreciable for a period of approximately 60 days after their first appearance. There also was no major increase in total iodine content in the glands at that time. As gestation progressed, however, the rate of follicular development clearly increased, as did the amounts of colloidal material in the lumina of the follicles, and the intracellular colloid droplets in the follicular epithelium. This intracellular activity accelerated notably during the last 2-2½ months of pregnancy. At that time, fetal thyroids reached almost complete development and a distinct increase in total iodine content was recorded. The epithelial cells which now had attained their maximum size were mostly columnar. Nuclei were round or oval in shape and vesicular in character. The apical portion of the cytoplasm of the follicular cells of many follicles was filled with numerous discrete colloid droplets (figs. 7 and 8). These figures also show the manner in which colloid droplets pass into the follicular cavity. This picture of secretory activity was not unlike that described by De Robertis in his studies on colloid secretion by the follicular epithelium (1941, 1942, and 1949).

DISCUSSION

In fetuses ranging from 53 to 70 days in age, the thyroid contained about three mg. per cent thyroxine and about 17 mg. per cent total iodine. In contrast, the concentrations of these two iodine fractions in the livers of three fetuses aged 62, 68, and 70 days—though too low to permit an accurate quantitative measure—were less than one-tenth those of their respective thyroid glands (Wolff and Nichols, 1949).

EXPLANATION OF PLATES

Photomicrographs were taken with 4 mm. objective (figs. 1 and 2), with oil immersion objective (figs. 3 to 8), and with 16 mm. objectives (figs. 9 to 16). Magnification $\times 400$, $\times 750$, and $\times 150$ respectively.

FIG. 1. An area from the central region of the thyroid gland of 63-day-old calf fetus. Note the arrangement of cells into quite regular cords. No intracellular colloid or follicles are present.

FIG. 2. An area from the thyroid gland of 78-day-old calf fetus. Note a few very young follicles. Also note that the cord arrangement became less regular.

The following figures (3 to 6) show initial stages of development of the follicles.

FIG. 3. Individual colloid droplets stainable with aniline blue or orange G can be seen in the cytoplasm (arrow).

FIG. 4. First drop of colloid located between two oblong, neighboring cells of the cord (arrow).

FIG. 5. Young follicle showing individual colloid drops not yet coalesced. Also note that one droplet of colloid is passing from the apical region of the cell into the lumen; note indented nucleus.

FIG. 6. Young follicle having many characteristics shown in fig. 5 but containing faintly vacuolated, irregular mass of fusing colloid droplets.

FIG. 7. Follicular epithelium from near-term gland showing two cells apical portions of which are ready to be cast off into the lumen.

FIG. 8. High columnar epithelium from the thyroid of fetus near-term showing massive accumulation of stainable colloid droplets in the apical zone of the cytoplasm and their passage into the lumen.

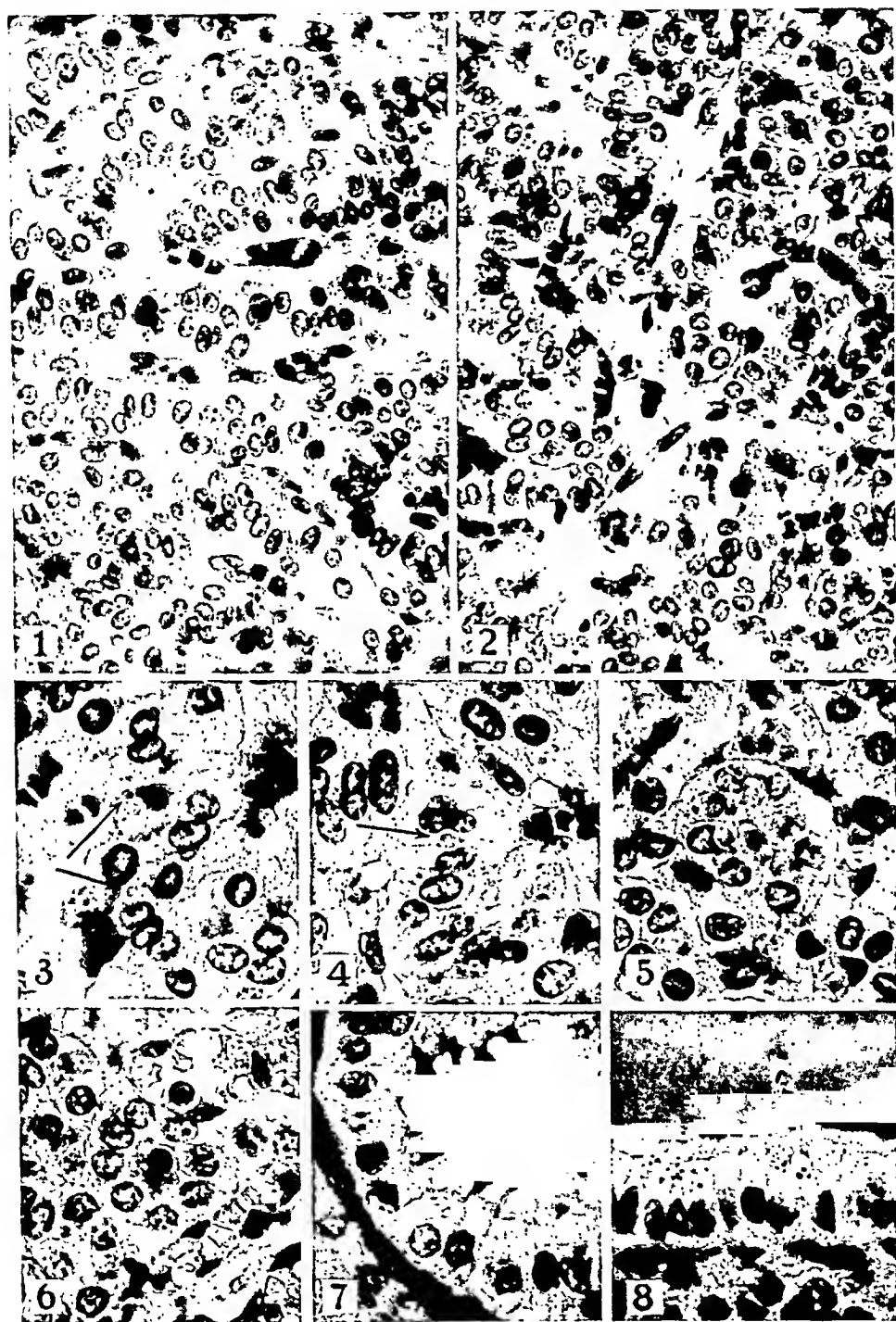


PLATE I

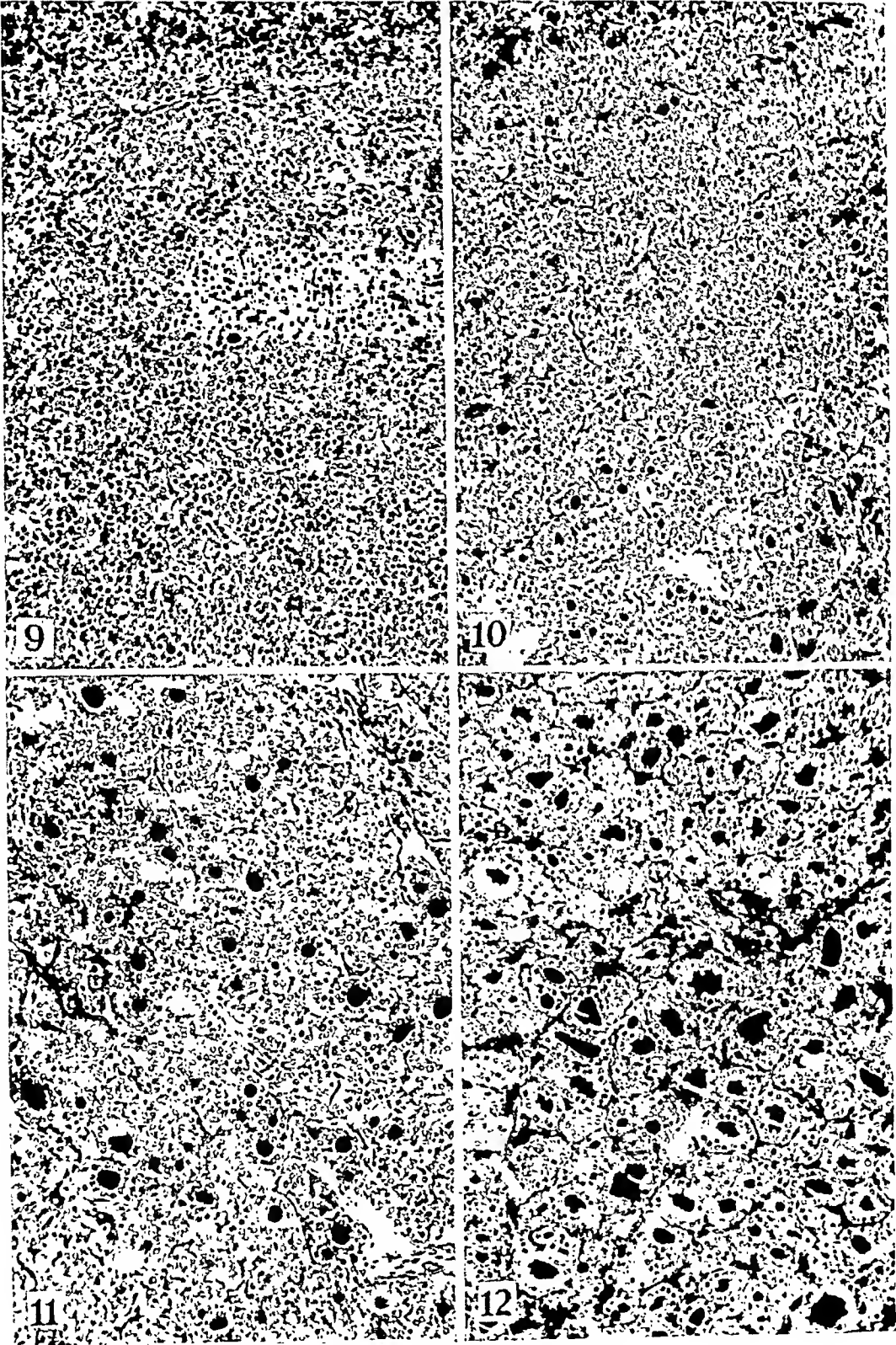


PLATE II

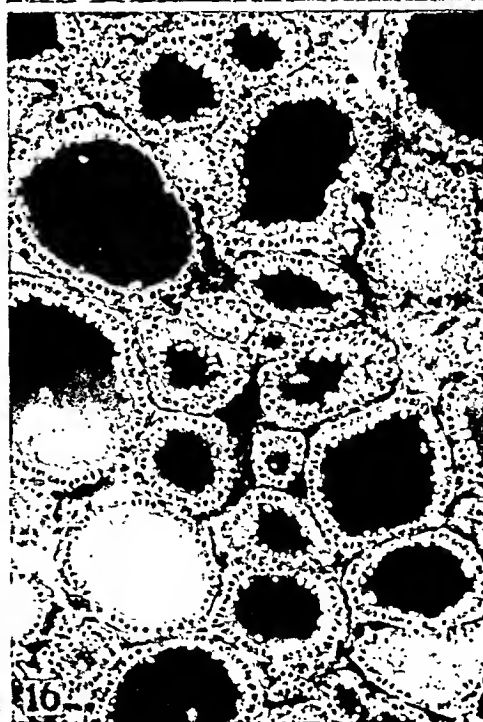
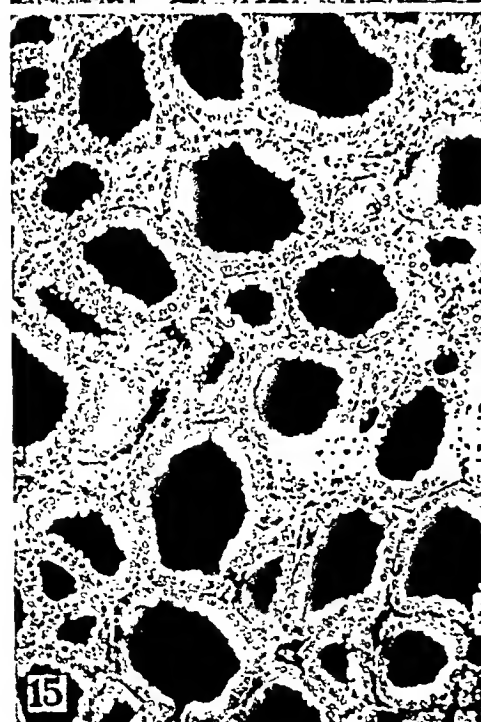
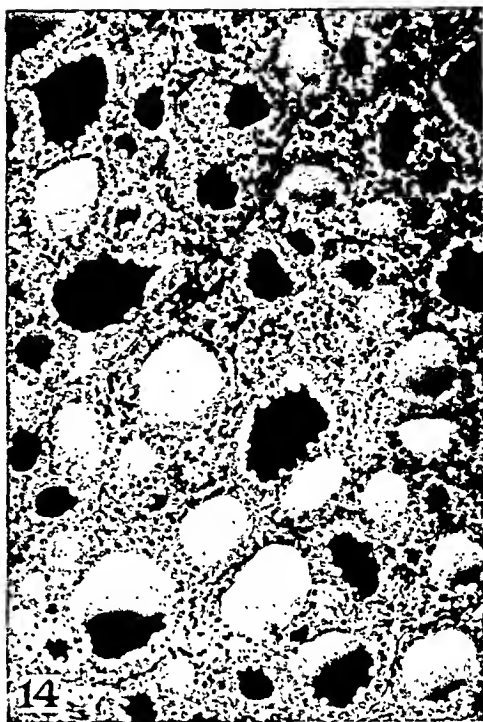
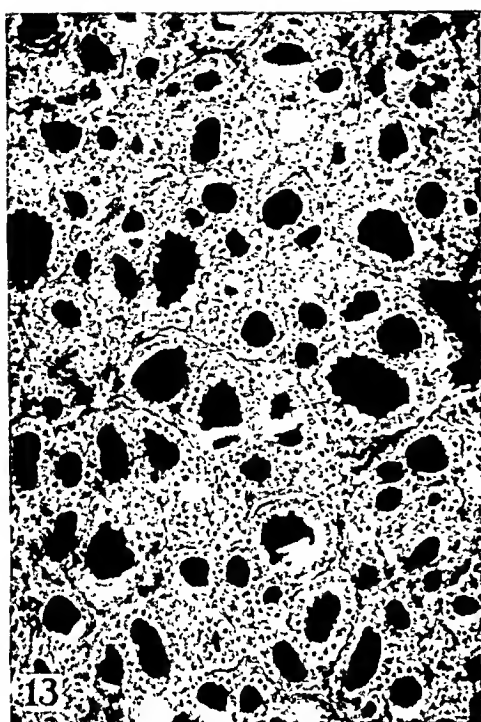


PLATE III

FIGS. 9 TO 12 ON PLATE II AND FIGS. 13 TO 16 ON PLATE III ARE PHOTOMICROGRAPHS REPRESENTING THYROIDS OF CALVES, 88, 90, 108, 125, 135, 155, 175, AND 254 DAYS OF INTRA-UTERINE LIFE RESPECTIVELY. NOTE PROGRESSIVE NUMERICAL INCREASE OF TYPICAL THYROID FOLLICLES AND THEIR DEVELOPMENT ACCOMPANIED BY GROWTH AND DEVELOPMENT OF FOLLICULAR EPITHELIUM. ALSO NOTE GRADUAL DIMINUTION OF INTERFOLLICULAR NESTS OF CELLS.

Thus concentration and storage of organic iodine in the fetal thyroid tissue takes place not only while the gland is still devoid of its characteristic architecture, but even prior to any histological manifestation of this specific function (intracellular colloid droplets). Follicular arrangement of thyroid epithelium is, therefore, not a prerequisite to iodine storage by this tissue.

Similar findings have been reported for the fetal pig by Rankin (1941) who detected thyroxine and diiodotyrosine in the thyroids of 52-day-old fetuses (gestation period 114 days) at a time when their thyroids contained neither follicles nor colloid.

Hogben and Crew (1923) reported follicle differentiation and minute colloid masses at three months in bovine fetal glands. In the same species, Aron (1931) found small amounts of stainable colloid at 15-18 cm. stage (corresponding to about 90 days of pregnancy as calculated here), whereas Abbott and Prendergast (1937) described colloid and follicle formation during the second month of life. In our series of calf thyroids, colloid was first detected *within the cytoplasm* of individual cells and *in the lumen* of very young follicles on the 75th day of intrauterine life.

The following may be considered as the first stages of the histological manifestation of secretory activity in early fetal thyroids: a) the appearance of intracellular colloid droplets and beginning of follicle formation in the glands of about 75- to 88-day-old fetuses; b) the presence of completely formed, although very small, follicles at approximately 90 days. In spite of this definite advance in their histological development, the fetal thyroids showed no major changes in the amounts of iodine stored, either during this period or in the following few weeks.

In the following months (from 120 to 205 days), the increase in the concentration of organic iodine was accompanied by the gradual histological development shown in figs. 12 to 15. Histological manifestations of activity, as judged by production and storage of colloid, in glands of these age groups differed only in degree and not in general character. The average values for total iodine were 20.0, 32.6, 34.1, and 67.0 mg. per cent for the groups represented by photographs 12 to 15, respectively. In the final months of gestation, total organic iodine content reached its maximum, averaging now 118 mg. per cent. This increase in storage capacity of the thyroid gland was accompanied by a very marked rise of functional activity of the follicular cells.

SUMMARY

Thyroxine-like and other organically bound iodine compounds appear in the fetal calf thyroid between the 53d and 70th days, a time prior to the appearance of histologically detectable intracellular colloid or follicle formation, which takes place at 75-88 days. Since the livers of fetuses aged 62-70 days contain practically no iodine,

the presence of these organic iodine compounds was considered a manifestation of iodine storage by the gland.

From the 75th to 118th day, the predominating features of the developing thyroid were the appearance of colloid and differentiation of the parenchyma without a pronounced increase in the iodine content of the gland.

Between the fourth and seventh month, gradual development of the fetal thyroid gland was accompanied by a steady increase in its organic iodine content.

An appreciable increase in organic iodine content occurred near term, and was accompanied by definite histological manifestations of increased functional activity of the follicular epithelium.

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THE NECESSITY OF TESTIS AND THYROID HORMONES FOR THE MAINTENANCE OF THE SEROUS TUBULES OF THE SUB- MAXILLARY GLAND IN THE MALE RAT

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SINCE the time, about a century ago, when research on the salivary glands began in earnest, most of the investigations dealing with these organs indicated that they were under the control of the nervous system (Babkin, 1944). Therefore, it came as a surprise to learn that the male sex hormone plays an important role in the maintenance of the serous tubules of the submaxillary glands of mice. This was first suspected by Lacassagne (1940a) when he found that this structure was far better developed in male than in female mice. Later he showed that the extirpation of the testes produced the atrophy of the serous tubules, while testosterone injections restored them to a normal condition (Lacassagne, 1940b). These effects were accompanied by changes in the enzymatic content of saliva. Thus, the ability of saliva to liberate glucose from starch was greater in males than in females, decreased by castration in males, and increased by testosterone injections in females (Raynaud and Rebeyrotte, 1949a, 1949b).

A complication was introduced by the finding that hypophysectomy produced a more pronounced atrophy of the serous tubules than castration (Lacassagne and Chamorro, 1940). This was explained by Raynaud (1946, 1947) on the assumption that cortico-adrenal hormones also can stimulate the serous tubules to some extent. Thus, after hypophysectomy, the absence of both adrenal and testicular secretions would account for the more complete atrophy of the tubules than after castration. However, the data of this author indicated that the role of the adrenals was only slight if any. Other endocrines might, therefore, be involved.

A role of the thyroid gland seemed likely, since in the rat thyroidectomy produces an atrophy of the serous tubules of the submaxillary gland (Leblond and Grad, 1948a). To study the roles of testis and thyroid, these two glands were removed and substitution therapy with testosterone or thyroxine or both was instituted. In this manner, it was demonstrated that a joint action of these hormones is necessary to maintain a normal development of the serous tubules in the submaxillary gland of the rat.

METHODS

The submaxillary gland of the adult male rat is rather complex; the acini, which may be classified as mucous,¹ are joined by short intervalated ducts to tubules lined by cells full of serous granules (Figs. 1 and 2). These serous tubules in turn empty into excretory ducts. Both tubules and ducts stand out in the sections because of their acidophilic properties, but the tubules may be differentiated from the ducts by their smaller lumen, the basal position of the nuclei in their cells and especially the presence of numerous granules in their cytoplasm.

Preliminary experiments were designed in order to examine how the serous tubules were affected by feeding and fasting, by age and sex and by thyroidectomy. The results of this work led to the main series of experiments, in which the effect of testosterone and thyroxine were examined in castrated-thyroidectomized rats.

PRELIMINARY EXPERIMENTS

The influence of feeding and fasting was examined in six groups of adult male rats which were fasted from 11 P.M. one evening to 8 A.M. the next

TABLE 1. THE EFFECT OF FEEDING AND FASTING ON THE SEROUS TUBULES OF THE SUBMAXILLARY GLAND OF THE MALE RAT

Hours of fast	Average body weights (gm)		Average diameter of serous tubules (micra)
	Initial	Final	
0	274	—	45.7 ± 2.0
2	267	—	51.0 ± 1.8
4	267	—	47.0 ± 2.3
24	263	273	48.0 ± 2.0
120	258	286	44.7 ± 0.2

Statistical Analysis: Differences between these values were not significant, except between the 0 and 120 hour groups, $P=0.02$. The difference between the 0 and 2 hour groups, $P=0.07$, was near the borderline of accepted significance.

morning, when they were fed Purina for chow ad libitum for one hour. One group of 4 animals was sacrificed at the end of this feeding period. The other animals were then fasted again, and sacrificed in groups of 4 or 5 animals at 2, 4, 24 and 120 hours later. The submaxillary glands of the animals were fixed in Spss, sectioned and stained with hematoxylin-eosin. The diameters of the serous tubules were measured in the sections with the help of a micrometer placed inside the ocular of the microscope. The diameter was taken as the maximal width of each tubule, that is to say, the longest perpendicular to the longest axis of the cross sections of a tubule. Ten measurements were averaged per animal; grand averages were then calculated for each group and reported in Table 1.

¹ The acini of the submaxillary gland of the adult male rat, when fixed in biobromate-formol, give an intense periodic acid-Schiff reaction and stained metachromatically with toluidin blue. They were, therefore, considered as being mucous and will be thus referred to in the present article.

It must, however, be noted that Sacramento (1933) and many others considered that these acini did not consist of true mucous cells, but rather of "special serous cells" or "mucoprotein cells."

The effect of age and sex was examined by measuring the diameter of the serous tubules in the submaxillary glands of the following groups of rats: 1) Five males and two females, 10 day old; 2) four females and four males, 39 day old; 3) nine males and nine females, 150 day old. The animals were fed on Purina fox chow (with the exception of the suckling 10 day old ones). They were sacrificed with chloroform. The submaxillary of the animals of groups 1 and 2 were fixed in Orth and stained with hematoxylin-eosin. The animals of group 3 were the normal controls of the next experiment. The diameters of the serous tubules were measured as in the previous experiment and reported in Table 2.

The effect of thyroidectomy and thyroxine treatment was examined in the following groups of rats, which all weighed less than 45 grams at the time of operation: 1) nine normal control males; 2) sixteen thyroidectomized males; 3) nine normal control females; 4) nine thyroidectomized females. It may be

TABLE 2. THE EFFECT OF AGE AND SEX ON THE SEROUS TUBULES OF THE SUBMAXILLARY GLAND OF THE RAT

Age (Days)	Average diameter of the serous tubules (miera)	
	Males	Females
10	22.3 \pm 0.52	21.3 \pm 0.58
39	31.9 \pm 0.61	29.4 \pm 0.45
150	45.8 \pm 0.48	42.3 \pm 0.35

Statistical Analysis: All differences between any two groups (except between 10 day old males and females) are highly significant ($P < 0.001$).

noted that thyroid removal included the ablation of the parathyroid glands.

The animals were then fed on Purina fox chow and left untreated for the next 16 weeks. At that time, the thyroidectomized animals were subdivided into two sub-groups, one of which remained untreated, while the other received 7.5 micrograms of sodium-DL-thyroxine twice daily for the next two weeks. All animals were then sacrificed with chloroform. The submaxillary glands were fixed in Susa and stained with hematoxylin-eosin. The diameters

EXPLANATION OF PLATE I

FIG. 1. A section of submaxillary gland of normal adult male rat. The section was fixed in Susa, and stained with hematoxylin-eosin. $\times 500$

This shows a duct (upper left hand side) leaving two tubules (lower centre and right), the remainder of the field being made up of mucous acini. This figure shows some of the differences between serous tubules and ducts: viz., the tubules have more basally located nuelei, narrower lumina and more granular cytoplasm.

FIG. 2. A section of submaxillary gland of normal adult male rat. The section was fixed in Susa, and stained with H & E. $\times 1,100$

A serous tubule full of prominent granules of variable size is shown.

FIG. 3. Submaxillary gland of adult male rat fasted for 9 hours, then fed Purina fox chow for one hour, and killed immediately thereafter with chloroform. Susa fixation, H & E stain. $\times 500$.

Granules are abundant in the serous tubules, although less than in those of Fig. 4.

FIG. 4. Submaxillary of adult male rat fasted for 9 hours, then fed Purina fox chow for one hour, and killed eight hours later. Susa fixation, H & E stain. $\times 500$.

Note that there are somewhat more granules present in the serous tubules than in those of Fig. 3.

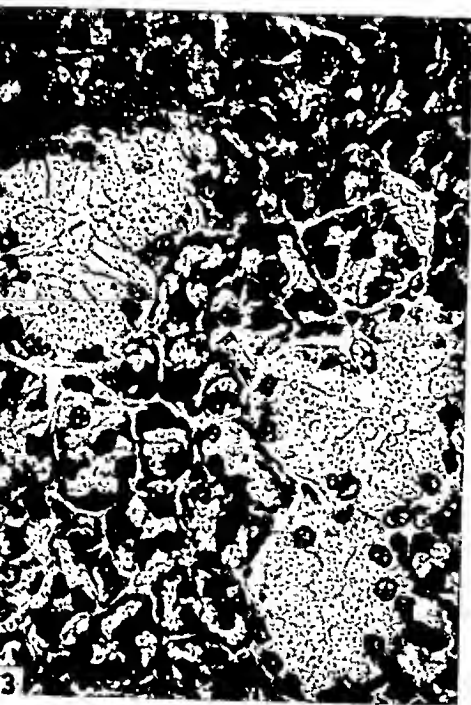


PLATE I

of the serous tubules were measured by ocular micrometer as indicated above and reported in Table 3.

MAIN EXPERIMENT

The joint action of testosterone and thyroxine was examined in an experi-

TABLE 3. THE EFFECT OF THYROIDECTOMY AND THYROXINE-TREATMENT ON THE SEROUS TUBULES OF THE SUBMAXILLARY GLAND OF THE RAT

	Average diameter of serous tubules (micra)	
	Males	Females
Normal Controls	45.8 ± 0.48	42.3 ± 0.35
Thyroidectomized	38.7 ± 0.71	33.5 ± 0.45
Thyroidectomized + Thyroxine	43.9 ± 0.68	37.7 ± 0.52

Statistical Analysis: All the differences between animals of different sexes receiving the same treatment, or of the same sex receiving different treatment were highly significant ($P < 0.001$) except that the difference between the normal males and the thyroxine-treated thyroidectomized males was reliable to a probability of 0.05 and the difference between the latter group and the thyroidectomized males was reliable to a 0.01 probability.

TABLE 4. EFFECT OF TESTOSTERONE AND THYROXINE ON CASTRATED-THYROIDECTOMIZED RATS

		Control	Testosterone	Thyroxine	Testosterone and Thyroxine
<i>Number of Animals at the End of the Experiment</i>		8	6	7	8
Body Weight (gm.)	Initial	175	180	168	175
	Final	168	163	213	206
Oxygen Consumption (cc./hr./100 sq. cm.)	Initial	82	82	82	81
	Final	58	58	79	74
Heart Rate (beats per minute)	Initial	366	364	368	355
	Final	329	314	374	391
Seminal Vesicles	Weight (mg.)	58	856	66	754
Submaxillary and Sublingual	Weight (mg.)	350	366	436	536
	Relative weight (mg./100 gm. body wt.)	209	226	206	257
Kidney	Weight (mg.)	1030	1107	1548	1825
	Relative weight (mg./100 gm. body wt.)	613	681	730	890
Heart	Weight (mg.)	569	581	826	878
	Relative weight (mg./100 gm. body wt.)	340	348	392	429
Hypophysis	Weight (mg.)	12.9	8.1	9.7	6.6
	Relative weight (mg./100 gm. body wt.)	7.7	4.9	4.7	3.2
	Acidophiles	Absent	Absent	Normal	Normal
	Basophiles	Increased	Increased	Increased	Normal

Statistical Analysis of Relative Organ Weights: P Values

	Control vs. Testosterone	Control vs. Thyroxine	Control vs. Testosterone and Thyroxine	Testosterone vs. Thyroxine	Testosterone vs. Testosterone and Thyroxine	Thyroxine vs. Testosterone and Thyroxine
Submaxillary and Sublingual (mg./100 gm. body wt.)	0.298	0.800	0.001	0.261	0.162	0.010
Kidney (mg./100 gm. body wt.)	0.167	0.018	0.001	0.288	0.001	0.008
Heart (mg./100 gm. body wt.)	0.700	0.020	0.001	0.090	0.008	0.080
Hypophysis (mg./100 gm. body wt.)	0.001	0.001	0.001	0.700	0.009	0.009

ment including 4 groups of 10 young adult male rats each, all of which had been castrated and thyroidectomized. Starting from the day after operation, the animals were injected twice daily as follows: 1) A control group given 0.1 cc. of a saline solution containing 1% duponol C; 2) a testosterone group given 0.25 milligrams of free testosterone in 0.1 cc. of a saline solution containing 1% duponol C. Some heating was necessary to obtain a satisfactory suspension of testosterone in the duponol solution; 3) a thyroxine group given 3 micrograms of DL-sodium thyroxine in 0.1 cc. of water; 4) a testos-

TABLE 5. THE EFFECT OF TESTOSTERONE AND THYROXINE ON THE DIAMETER OF THE SEROUS TUBULES OF THE SUBMAXILLARY GLAND

	Control	Testosterone	Thyroxine	Testosterone and Thyroxine
Diameter of Serous Tubules (microns)	26.3	28.1	29.8	36.0
Percent Increases of Tubular Width over Controls		7.1	13.3	37.2
<i>Statistical Analysis: P Values</i>				
	Control vs Testosterone	Control vs Thyroxine	Control vs Testosterone and Thyroxine	Testosterone vs Thyroxine
Diameter of Serous Tubules (microns)	0.103	.009	.001	0.278
				.001
				.001

terone-thyroxine group receiving the 2 hormones at the doses indicated above, care being taken to inject testosterone on the one side of the body and thyroxine on the other throughout the experiment.

The animals were given a 1% solution of calcium lactate instead of drinking water in order to reduce the number of deaths from tetany. Their diet consisted of the following: corn meal, 70.9%; wheat gluten, 16.4%; dried brewer's yeast, 9.0%; pig liver, 1.9%; sodium chloride, 0.9% and calcium carbonate, 0.9%. It was found that this diet, which is a modification of Remington's iodine deficient diet No. 342 (Levine, Remington and von Kolnitz, 1933), made it possible to obtain thyroidectomized animals that did not gain weight and showed a complete absence of acidophilic cells in the hypophysis: complete absence of acidophiles in Susa fixed hypophyses of thyroidectomized rats is diagnostic of a successful thyroid ablation (Leblond, 1944). These results were not easily obtained with commercial feeds such as the Purina fox chow. On the other hand, thyroxine treatment produced a satisfactory growth in the castrated-thyroidectomized animals given the iodine deficient diet, as may be seen from the body weights in Table 4.

The animals were weighed at weekly intervals in the course of the experiment. Their oxygen consumption and heart rate were estimated by a method previously described (Leblond and Grad, 1948b).

The animals were sacrificed with chloroform 47 days after the hormone treatment had been instituted. A number of organs were fixed in Orth fluid,

weighed, sectioned and stained with hematoxylin-eosin. Table 4 includes the observed organ weights and the organ weights corrected for 100 grams of body weight. In the case of the hypophyses, which were fixed in Susa, the weights were reported along with the results of a microscopic search for acidophiles and thyroidectomy cells in the anterior lobe (Table 4).

The submaxillary glands, with the sublingual glands attached, were fixed in Orth, weighed and prepared like the other tissues. The diameters of the serous tubules were estimated as above (Table 5). A more precise histometric method was also used, in which the relative weights of the serous tubules and other parts of the glands were estimated from enlarged projections of the sections. The body tube of the microscope was placed in a horizontal position, 8" above the table. A 45° projection prism (Bausch and Lomb) was adapted to the eyepiece to project the image on the table at a magnification of 430 times. For each animal, three fields were picked at random on the periphery of a section of the gland, and the outlines of the serous tubules and ducts were drawn on paper. The parts of the projection which did not represent either serous tubules or ducts consisted mostly of mucous acini with a few blood vessels, some connective tissue, and interstitial spaces. This was described as "remainder." Relative weights were estimated by cutting out of the paper the images of the various glands components and weighing them separately (Table 6). The percentage of the space occupied by each component was computed from these figures (Table 7).

The studies just reported were directed towards measuring any change in the size of the serous tubules under the different hormonal conditions. In the series of measurements now to be described, an attempt was made to determine whether the variation in amount of tubular tissue was accompanied by a change in the number of cells. To this end, the number of serous-tubule nuclei per field in 6 μ sections was counted for each animal, the average of which was reported in Table 8 as crude number of nuclei. The average diameter of the nuclei was also estimated and reported in the same table. Both measurements were performed on projections obtained as described above, but this time the magnification was 928. These data made it possible to calculate the true number of nuclei per field by Abercrombie's method (1946). On the assumption that the density of the tissue is equal to unity, the weight of the field could be estimated by multiplying its area by the thickness of the section. Knowing the number of nuclei per field, the weight of

EXPLANATION OF PLATE II

Submaxillary glands of 150 day old rats. The pictures on the left are from females; those on the right are from males. All photographs refer to Susa fixed, H & E stained sections. $\times 60$.

FIG. 5. Intact female. The pale areas are the serous tubules.

FIG. 6. Intact male. The tubules are larger here than in females (Fig. 5).

FIG. 7. Thyroidectomized female. The tubules are smaller than those of normal females (Fig. 5).

FIG. 8. Thyroidectomized male. The tubules are smaller than those of normal males (Fig. 6), but somewhat larger than those of thyroidectomized females (Fig. 7).

FIG. 9. Thyroidectomized female treated with 15 micrograms of thyroxine daily for two weeks before autopsy. The tubules are larger than in untreated thyroidectomized females (Fig. 7).

FIG. 10. Thyroidectomized male treated with 15 micrograms of thyroxine daily for two weeks before autopsy. The tubules are larger than in untreated thyroidectomized males (Fig. 8).

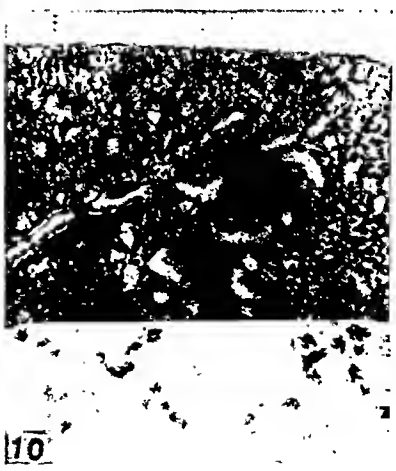
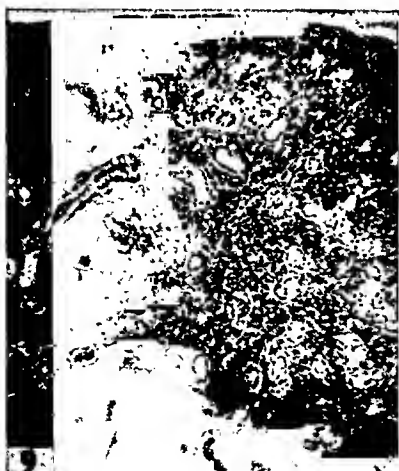
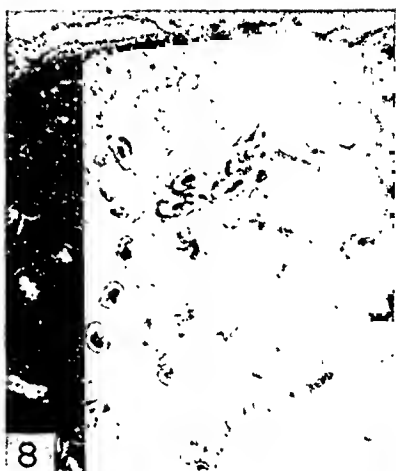
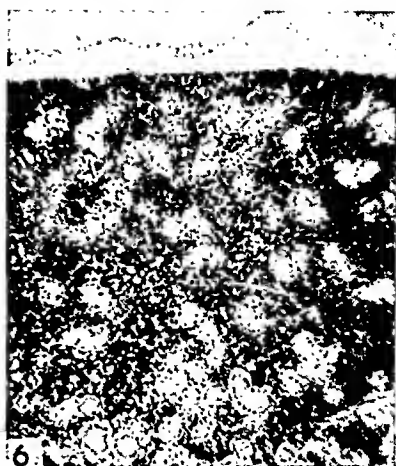


PLATE II

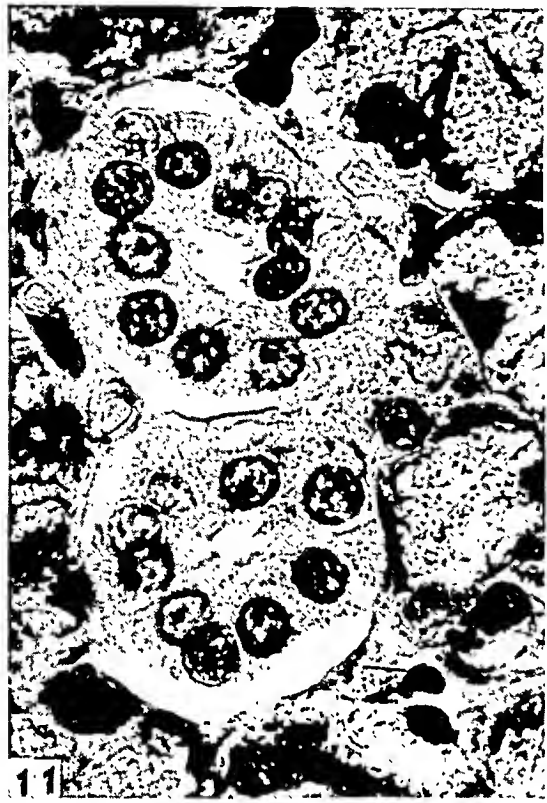


PLATE III

TABLE 6. THE EFFECT OF TESTOSTERONE AND THYROXINE ON THE RELATIVE WEIGHTS OF THE SUBMAXILLARY GLAND COMPONENTS, AS CALCULATED FROM PROJECTIONS OF MICROSCOPIC IMAGES

	Weights of Paper Projections (mg.)			
	Control	Testosterone	Thyroxine	Testosterone and Thyroxine
Serous Tubules	773.5	1071.6	1078.9	1301.6
Ducts	262.6	182.4	284.0	204.3
Remainder	4619.0	4316.6	4241.6	4154.3
Total	5655.1	5570.6	5604.5	5660.2

Statistical Analysis: P Values

	Control vs Testosterone	Control vs Thyroxine	Control vs Testosterone and Thyroxine	Testosterone vs Testosterone and Thyroxine	Thyroxine vs Testosterone and Thyroxine
Serous Tubules	0.158	0.097	0.009	0.255	0.252
Ducts	0.279	0.800	0.390	0.800	0.278
Remainder	0.100	0.047	0.019	0.378	0.700

TABLE 7. PERCENTAGE OF SUBMAXILLARY GLAND OCCUPIED BY SEROUS TUBULES, DUCTS, ETC., AS CALCULATED FROM MEASUREMENTS OF PROJECTIONS OF THE MICROSCOPIC IMAGES

	Control	Testosterone	Thyroxine	Testosterone and Thyroxine
Serous Tubules	13.7	19.1	19.3	23.0
Ducts	4.6	3.3	5.1	3.6
Remainder	81.7	77.6	75.6	73.4

each field, and the weight of the corresponding gland, it was possible to calculate the number of serous-tubule nuclei in the whole gland² (Table 8).

² The calculated number of nuclei was only an approximation, since the weight of the gland used in this calculation was the combined weight of the submaxillary and sublingual glands. However, the sublingual glands were considered small enough in regard to the submaxillary to justify neglecting them.

EXPLANATION OF PLATE III

Submaxillary glands from the main experiment. Orth fixation. H & E stain. $\times 1,100$

FIG. 11. Control castrated-thyroidectomized male rat. Note the small size of the tubules and the complete lack of granules.

FIG. 12. Castrated-thyroidectomized male rat treated with testosterone. Note the slightly larger size of the tubules although granules are still not visible.

FIG. 13. Castrated-thyroidectomized male rat treated with thyroxine. The tubule is now larger than those in Figs. 11 and 12. Note also the mitotic figure. In common with the tubules of Figs. 11 and 12, there is still a deficiency of granules in the cells as well as a relatively central position of the nuclei.

FIG. 14. Castrated-thyroidectomized male rat treated with testosterone and thyroxine. Note the great increase in the width of the tubules over those of other groups, as well as the loading of the cytoplasm of the cells with granules which force the nuclei to a basal position. From a consideration of Figs. 11 to 14 inclusive, it is obvious that the tubules increase in size largely through the increase in the height of the cell and not through the increase of the lumen.

TABLE 8. THE EFFECT OF TESTOSTERONE AND THYROXINE ON THE NUMBER AND SIZE OF THE NUCLEI IN THE SEROUS TUBULES OF THE SUBMAXILLARY GLAND OF THE RAT

	Control	Testosterone	Thyroxine	Testosterone and Thyroxine
Crude Number of Nuclei per Field	83.9	77.7	82.5	68.5
True Number of Nuclei per Field	44.9	41.6	43.8	36.4
Diameter of Nucleus (micra)	5.2	5.1	5.3	5.3
Calculated Number of serous Nuclei in the Whole Gland ($\times 10^6$)	70.4	68.2	85.5	87.3

Statistical Analysis: P Values

	Control vs Testosterone	Control vs Thyroxine	Control vs Testosterone and Thyroxine	Testosterone vs Thyroxine	Testosterone vs Thyroxine and Testosterone	Thyroxine vs Thyroxine and Testosterone
Crude Number of Nuclei per Field	ca. 1.000	ca. 0.700	0.355	0.720	0.414	0.668
Diameter of Nucleus	ca. 0.600	ca. 0.565	ca. 0.600	0.350	0.363	ca. 1.000

In all experiments, standard errors were determined by analyses of variance, and significant differences found by "t" tests. According to the usual conventions, a *P* value lower than 0.05 was considered to be significant, while values lower than 0.01 were considered highly significant. Values lower than 0.001 were reported in the Tables as being equal to 0.001.

RESULTS

PRELIMINARY EXPERIMENTS

The effect of feeding and fasting on the serous tubules was only moderate. Histological examination revealed the presence of abundant granules in the serous cells of all groups. Some decrease in the amount of granules seemed to occur in the zero-hour group (Fig. 3), that is, at the end of a period of active feeding, while the number of granules appeared greater at 2 and 8 hours after the beginning of the fast (Fig. 4). However, measurements of the diameter of the serous tubules showed no significant differences between the zero-hour and the 2 or 8 hour groups (Table 1). The diameter of the tubules and the number of granules were definitely decreased in the group fasted for 120 hours (Table 1). It may be noted that in all groups, most of the ducts contained a rather large amount of secretion. No obvious differences in the nature and amount of duct content of the various groups could be observed.

The effect of age manifested itself by a gradual increase of the diameter of the serous tubules with increasing age. This increase was more pronounced between the 10th and 39th day, than between the 39th and 150th day (Table 2). Well developed secretion granules were present only in the oldest group.

The effect of sex was indicated by a greater diameter of the serous tubules in males than in females at 39 and 150 days. Similarly, in the next experiment (Table 3), males had larger tubules than females, whether the animals were intact, thyroidectomized or treated with thyroxine (Fig. 5-10). Such a result pointed to a role of the male hormone on the growth of the serous tubules.

The effect of thyroidectomy was also brought out by this experiment (Tables 3, Figs. 5-8), since in both sexes this operation reduced the size of the serous tubules. Furthermore, the tubules were partly restored by administration of thyroxine (Figs. 9-10). Histological examination under high power showed an almost complete absence of serous granules after thyroidectomy, while these were prominent in normal and thyroxine-treated animals. However, there was a possibility that thyroidectomy acted on the submaxillary glands by reducing the activity of the gonads and decreasing their secretion of testosterone. Another experiment was, therefore, necessary to separate the respective roles of thyroids and gonads.

THE MAIN EXPERIMENT

The results of the main experiment (Table 4) were interpreted on the basis of an absence of hormonal secretion by testis and thyroid.

The effectiveness of castration was demonstrated by the atrophy of the seminal vesicles in the control and thyroxine treated groups. Conversely, the repair of castration symptoms by testosterone was demonstrated by the enlargement of the seminal vesicles in the two other groups.

The completeness of the thyroidectomy was demonstrated in the control and testosterone-treated group by the lack of gain in body weight, the decrease in oxygen consumption and heart rate, as well as by the complete absence of acidophiles and the presence of thyroidectomy cells in the hypophysis. Conversely, the action of thyroxine was demonstrated in the two remaining groups by the gain in body weight, the increase in oxygen consumption and heart rate, and the abundance of acidophiles in the hypophysis.

Testosterone alone had no statistically significant effect on the diameters (Table 5) and surface areas (Table 6 and 7) of the serous tubules. Thyroxine alone had a slight effect that was statistically significant only in the case of the diameters (Table 5). When both testosterone and thyroxine were administered jointly, there was a highly significant increase in both the diameters (Table 5) and surface areas (Table 6) of the serous tubules. It may be noted that both the diameters and the surface area were indices of cell size, since the lumina were small enough in all groups to be neglected (Figs. 11-14).

Microscopic examination revealed that the cells of the serous tubules in the operated control animals were shrunken and contained practically no granules (Fig. 11). In the animals treated with testosterone (Fig. 12) or thyroxine (Fig. 13), the cells appeared larger and

occasional granules were found. But the animals treated with both hormones had serous tubules distended with granules (Fig. 14), which pushed the nuclei against the basement membrane as in intact male rats.

The total number of nuclei in the serous tubules of the gland (Table 8) was not affected by testosterone alone, but was equally increased whether thyroxine was given alone or jointly with testosterone.

The joint action of thyroxine and testosterone was not confined to the submaxillary gland, but extended to the heart, kidney and hypophysis. In the case of heart and kidney, the moderate effect of testosterone alone was not statistically significant, but that of thyroxine alone was (Table 4). When testosterone was administered jointly with thyroxine, the increase in kidney weight became definitely greater than with thyroxine alone (Table 4), a fact already demonstrated by Masson and Romanchuck (1945). In fact, in the case of kidney and heart, the increase in weight due to the combined hormonal treatment was synergistic, that is to say, greater than the sum of the increases in weight due to each single hormonal treatment.

Testosterone and thyroxine together decreased the weight of the hypophysis more than either hormone alone (Table 4). Histological study of this gland revealed the presence of a typical thyroidectomy picture in the testosterone-treated group, since no acidophiles and many pale basophiles were found. A typical castration picture was noted in the thyroxine-treated group, since many acidophiles and more deeply stained basophiles were found. The animals given both hormones had normal-appearing hypophyses, while the untreated controls showed a combination of the signs of castration and thyroidectomy.

In contrast, the effect of testosterone on the seminal vesicles was less intense in the presence of thyroxine than in its absence, as shown by a significantly ($P=0.008$) lower weight of the seminal vesicles after the combined hormonal treatment than after testosterone alone (Table 4). Masson and Romanchuck (1945) previously obtained the same results with daily doses of 60 micrograms of testosterone propionate and 10 to 40 micrograms of thyroxine. However, this effect was dependent on the dosage used, since later, Masson (1947) reported that 50 micrograms of testosterone propionate and 112.5 micrograms of thyroxine had a synergistic action on the weight of the seminal vesicles, thus confirming the earlier work of Caridroit and Arvy (1942).

DISCUSSION

Lacassagne showed that *testosterone* repairs the atrophy of the serous tubules brought about by castration in the male mouse (1940b). Frantz and Kirschbaum confirmed Lacassagne's results and

went so far as to consider the serous tubules as a more sensitive indicator of the presence of androgens than the seminal vesicles (1949a, b).

In an attempt to duplicate his results in rats, Lacassagne (1940c) concluded that the serous tubules in this species were also controlled by the male hormone. In agreement with this finding, the first results obtained in the present work showed a greater diameter of the tubules in male than in female rats, whether the animals were normal (Table 2), thyroidectomized or thyroidectomized and thyroxine treated

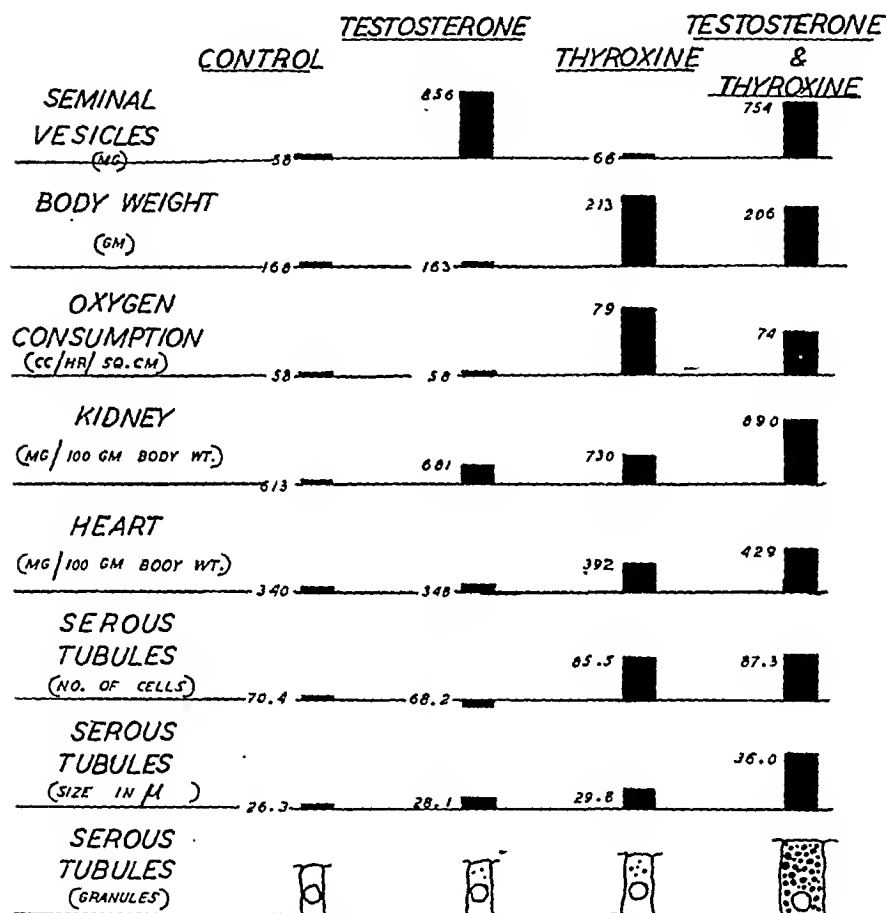


FIG. 15. Block diagram summarizing the effects of testosterone and/or thyroxine in castrated-thyroidectomized rats. In the case of each response the figure obtained for the controls was used as base line. The figures for seminal vesicle weight, body weight, oxygen consumption, kidney weight and heart weight were taken from Table 4. The figures for the number of cells in the serous tubules were taken from Table 8 and those for their size, from Table 5. Finally, a schematic drawing of the amount of granules in the cells of the serous tubules was added at the base of the chart. It is apparent that a synergistic action of testosterone and thyroxine is present only in the case of kidney weight, heart weight, and size and granule content of the serous tubules.

(Table 3). This sex difference had to be interpreted with caution, however, since the males, which had larger tubules than comparable females, also weighed more. Still, it suggested the possibility of an action of the male hormone on the serous tubules.

In the main experiment, in which the thyroid and testes of all animals had been removed, testosterone alone produced a large increase in the weight of the seminal vesicles, but had only a slight, statistically insignificant effect on the serous tubules (Fig. 15). In other words, testosterone had little or no action on the tubules, while its androgenic properties were fully evident. Therefore, the serous tubules did not behave as a secondary sex organ.

When *thyroxine* alone was administered, it raised the oxygen consumption to the maximum obtained in this experiment, but it caused only a slight increase in the size of the serous tubules (Fig. 15). This slight effect of thyroxine was significant when the criterion used was the diameter of the tubules (Table 5), but not when it was the surface area (Table 6). Furthermore, the serous granules were not restored to normal (Fig. 13). In contrast, thyroxine alone increased the number of serous cells to the maximum obtained in this experiment (Table 8). This response which paralleled that of oxygen consumption and body weight, seemed to be merely one more sign of the overall metabolic stimulation due to thyroxine.

While the two hormones given alone had little influence on the size and granule content of the serous tubules, the simultaneous administration of both *testosterone* and *thyroxine* produced a highly significant increase in the size of the tubules (Tables 5 and 6) and a massive accumulation of granules in their cells (Fig. 14). The combined effect of the hormones on the diameters of the tubules was synergistic, that is greater than the sum of the individual effects (Table 5). The formation of granules could also be considered as a synergistic response (Figs. 10-14). Of the end-organs listed in Fig. 15, only the kidney and heart weights showed a similar synergistic response to the two hormones.

Whether the participation of testosterone in the joint action on the serous tubules was exerted through its androgenic or other properties, and whether that of thyroxine was exerted through its metabolic-stimulating or other properties could not be definitely answered from this study.

While the results established the joint action of testosterone and thyroxine on the serous tubules of the castrated-thyroidectomized animals, the question arose as to whether a similar hormonal combination was responsible for the maintenance of these tubules in the normal male rat. In the first place, it could be assumed that testosterone is the hormone secreted by the rat testis (Moore, 1939), while thyroxine is secreted by the rat thyroid (Leblond and Gross, 1949). Secondly, the picture obtained by treatment of castrated-thyroidec-

tomized rats with these two hormones was similar to that found in the intact male rat. Therefore, it was concluded that under physiological conditions too, the joint action of these hormones may be held responsible for the maintenance of the serous tubules in the submaxillary gland of the rat.

In contrast to the profound influence of the testis and thyroid hormones on the serous tubules, the influence of *feeding and fasting* was surprisingly small (Table 1). The size of the tubules shrank significantly only after a 5-day fast. Even here hormones may have been involved, since a prolonged fasting was shown to decrease the activity of testis (Mulinos and Pomerantz, 1940) and thyroid (d'Angelo, Gordon and Charipper, 1949). Therefore, the direct influence of feeding and fasting—presumably exerted through the nervous supply of the gland—played no more than a minor role in maintaining the serous tubules.

The presence of granules in fed as well as in fasted animals, and the occurrence of secretion material in the lumen of the ducts in animals fasted for various lengths of time, suggested that some secretory activity of the gland was taking place more or less continuously, as already assumed by Babkin (1944) in the case of other digestive glands. Such a constant secretory activity might well be related to the continuous action of the testis and thyroid on the serous cells of the submaxillary gland.

SUMMARY

The object of this study was to examine the endocrine control of the serous tubules which, in addition to mucous acini, constitute the secretory portion of the submaxillary gland of the rat.

Preliminary experiments revealed that male rats had larger serous tubules than females (Table 2) and thyroidectomy decreased the size of the tubules in both sexes (Table 3). Therefore, both testis and thyroid seemed involved in the control of this structure. In order to assess the respective roles of these two glands, young adult male rats, which had been thyroidectomized and castrated, were treated daily for 47 days with 0.5 mg of testosterone or 6 micrograms of thyroxine or both, and the serous tubules were examined by histometric methods.

The results are as follows:

- 1) Control animals, in which testes and thyroids are extirpated, show an atrophy of the serous tubules.
- 2) Treatment of such animals with testosterone alone has little or no effect on the size and granule content of the cells of the serous tubules.
- 3) Treatment with thyroxine alone has little effect on the size and granule content of the cells of the serous tubules, but it restores the number of these cells to normal.

4) A combined treatment with both hormones restores a normal size and granule content as well as a normal number of the cells of the serous tubules (Figs. 11-14, Tables 5-8).

The testis and thyroid hormones in the doses indicated exert a synergistic action on kidney and heart as well as on the serous tubules of the submaxillary gland, but not on the male secondary sex organs, such as seminal vesicles, nor on the metabolic responses to thyroxine, such as the increase in oxygen consumption and body weight (Fig. 15).

In comparison with the major role of the testis and thyroid hormones in the maintenance of the serous tubules, nervous influences—such as those occurring in the course of feeding and fasting (Table 1)—have only a minor effect.

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THE EFFECT OF GROWTH HORMONE UPON LIVER AND KIDNEY D-AMINO ACID OXIDASE AND UPON MUSCLE SUCCINIC ACID DEHYDROGENASE

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SINCE enzymes are proteins, and may indeed constitute a major part of the protein in cells (Sumner and Somers, 1947) several relationships between them and the growth hormone are conceivable. As catalysts, tissue enzymes might be chemical mediators through which certain effects of the hormone are produced; as proteins, they may simply participate in any increase of body protein. In the case of bone phosphatase, marked changes in concentration which are readily correlated with known effects of the growth hormone on skeletal development are observed after hypophysectomy and after replacement therapy, and a functional relationship is indicated (Mathies and Gaebler, 1949). The concentrations of the two enzymes discussed in this paper do not change during induced growth, so there is no indication of a relationship to the growth hormone differing from that of other tissue proteins.

EXPERIMENTAL

Intact rats of two strains and both sexes, 6 to 10 months old, and Sprague-Dawley female hypophysectomized rats, 1 to 2 months old, were used under conditions of *ad lib.* and paired feeding as indicated in the tables.

D-amino acid oxidase was determined essentially as outlined by Axelrod, *et al.* (1940). One ml. of 16.7% (1:5) liver homogenate or 0.5 to 1.0 ml. of 9.1% (1:10) kidney homogenate was placed in a 15 ml. Warburg vessel. One ml. of 0.1 M pyrophosphate buffer of pH 8.3 was added, plus distilled water to give a total volume of 2 ml.; 0.5 ml. of 2 M DL-alanine in the same pyrophosphate buffer was added from the side-arm after equilibration. Other conditions were: air in the gas space, 0.2 ml. of 10% KOH in the inset, and a digestion temperature of 38.0° C. Determinations were carried out in duplicate and blanks were run in every instance. The oxygen uptake is expressed as three times the 20 minute uptake after correction for the blank.

Succinic acid dehydrogenase was assayed as described by Umbriet, *et al.* (1945), except that 0.5 ml. water was omitted, giving a final

TABLE 1. THE EFFECT OF GROWTH HORMONE ON D-AMINO ACID OXIDASE OF KIDNEY AND LIVER†

Experiment	No. of rats	Average body wt. at start of injection period in gm.	Average weight gained during injection period in gm.	Average terminal liver weight in gm.	Average terminal wt. of both kidneys in gm.	Average O ₂ uptake in μ l./hr.					
						Liver		Kidney			
						Per gm. tissue	Per mg. total N.	Per 100 gm.* body wt.	Per gm. tissue	Per mg. total N.	Per 100 gm.* body wt.
I. 8 month old Sprague-Dawley females, pair-fed. Given 3 mg. growth hormone (1485 R.U./gm.) per day/rat for 7 days.											
Treated	5	251	13	6.13	1.67	-258	-1387	-600	-1387	-876	-876
Controls	5	253	5	6.64	1.62	-384	-1619	-990	-1619	-1015	-1015
II. 6 month old "Edwards" females. <i>Ad lib.</i> fed. 6 mg. of above growth preparation given daily/rat for 6 days; 12 mg. on the 7th day.											
Treated	5	202	24	8.26	1.60	-565	-2370	-2060	-2370	-1680	-1680
Controls	5	208	1	7.34	1.53	-468	-1950	-1640	-1950	-1420	-1420
III. 8 month old Sprague-Dawley females, pair-fed. Given 6 mg. growth hormone (1740 R.U./gm.) per rat per day for 7 days; 12 mg. on the 8th day.											
Treated	5	256	21	8.44	1.91	-371	-10.28	-1131	-1850	-62.9	-1270
Controls	5	264	4	7.79	1.84	-242	-6.80	-704	-1780	-59.9	-1220
IV. 8 month old Sprague-Dawley females, pair-fed. Given 6 mg. growth hormone (1740 R.U./gm.) per rat per day for 7 days.											
Treated	4	244	12	7.20	1.69	-402	-2070	-1130	-2070	-1370	-1370
Controls	5	257	1	7.57	1.77	-293	-1720	-859	-1720	-1180	-1180
V. 8-10 month old Sprague-Dawley males. <i>Ad lib.</i> fed; 10 mg. growth hormone (1740 R.U./gm.) given per day per rat for 14 days. Fasted 24 hrs. before sacrifice.											
Treated	5	481	16†	10.96	2.79	-243	-6.09	-536	-1760	-56.0	-988
Controls	5	471	4†	11.13	2.79	-378	-9.22	-887	-2470	-77.2	-1450

* Calculated according to Miller (1948).

† Diet was Rockland rat diet (powdered for the paired-feeding experiments).

‡ Gain by the 13th day.

TABLE 2. TREATMENT OF HYPOPHYSECTOMIZED RATS WITH GROWTH HORMONE AND ITS EFFECT ON THEIR KIDNEY AND LIVER D-AMINO ACID OXIDASE

Experiment	No. of rats	Average body wt. at start of injection period in gm.	Average weight gained during injection period in gm.	Average terminal liver weight in gm.	Average terminal wt. of both kidneys in gm.	Average O ₂ uptake in μ l./hr.			
						Liver		Kidney	
						Per gm. tissue	Per mg. total N.	Per 100 gm. body wt.	Per gm. tissue
I. 2 month old Sprague-Dawley female rats. Diet of Bennett, <i>et al.</i> (1948). Hypophysectomized rats fed <i>ad lib.</i> ; controls pair-fed against them. Plateaued for 14 days after operation and then injected 1 mg. growth preparation, daily per rat for 17 days (1384 R.U./gm.). B1 fed against A1; B2 against A2.									
A. Hypophysectomized.									
1. Treated	6	154	53	6.80	1.00	-132	-3.98	-432	-1150
2. Controls	5	146	-1	4.65	0.75	-162	-4.46	-518	-1170
B. Normal.									
1. Treated	5	175	25	5.45	1.11	-234	-6.14	-639	-1297
2. Controls	5	165	-1	4.27	1.00	-200	-5.17	-518	-1350
*Same as above, except a 24-day injection period was used and all rats were pair-fed against group A2.									
II. A. Hypophysectomized.									
1. Treated	5	176	46	5.39	1.10	-135	-3.93	-326	
2. Controls	7	173	5	5.80	0.88	-135	-3.69	-430	
B. Normal.									
1. Treated	5	189	23	4.86	1.24	-234	-6.07	-536	
2. Controls	6	191	2	4.62	1.34	-234	-6.35	-560	

* Enzyme assays carried out on pooled samples.

volume of 2.5 ml. The succinate was added from a side-arm at zero time as recommended by Axelrod, *et al.* (1942) for muscle, and a single concentration of muscle (0.2 ml. of 16.7% or 1:5) was employed in the duplicates while 2 concentrations of cytochrome c (0.2 ml. and 0.3 ml. of the stock solution, 0.1 ml. being the amount at which O₂ uptakes plateaued) were used. Blanks were found to be unnecessary. The oxygen uptake is expressed as two times the 30 minute uptake.

The kidneys, liver, and muscle (tibialis anticus) were removed immediately after sacrifice and weighed. Tissues were analyzed on the day of sacrifice in most instances; those which were not immediately assayed were stored at -18° C. Homogenates were prepared in the usual manner and assayed immediately after preparation. Muscle was first disintegrated by freezing with liquid air in a steel mortar and pulverizing by a sharp blow on the pestle, before homogenizing.

Micro-Kjeldahl determinations were carried out according to Hiller, Plazin and Van Slyke (1948).

RESULTS

1. D-amino Acid Oxidase. As mentioned in a preliminary communication (Mathies, 1949) *in vitro* experiments gave no indication of any specific relationship between growth hormone and D-amino acid oxidase. Experiments summarized in Table 1 were carried out on mature white rats to determine whether an *in vivo* effect could be demonstrated. An hereditary difference may exist, for the "Edwards" animals have a greater oxidase content than the Sprague-Dawley strain. No significant sex difference is apparent. Within wide limits, individual variations in the content of this enzyme being high, the concentration of oxidase in the liver appears to parallel that of the kidney, averaging one fifth of the kidney concentration. Also, the total amount in the liver is approximately equal to that in the kidney. This would seem to be true for the mature animal, but not the immature animal, as lower liver values were obtained in the latter. There appears to be no significant alteration in the enzyme after injections of growth preparation. The concentration remained essentially unchanged, and it would seem that the enzyme was synthesized in proportion to the growth in tissue.

To demonstrate this more conclusively, larger weight changes were required. The growth response of immature hypophysectomized female rats was therefore investigated. Results in Table 2 indicate that no change in the concentration of the enzyme occurred in spite of the high rate of induced growth. Group IA showed an increase in kidney weight of 33%, while the concentration of oxidase remained the same. Evidently, the added tissue contained its normal complement of the enzyme. Liver weight responded similarly, and it is evident that oxidase was also synthesized here at a rate proportional to growth. Normal animals, treated and untreated (Table 2, groups

IB and IIB), have a higher concentration of the enzyme in the liver than treated and untreated hypophysectomized animals. This is probably related to the loss of the thyrotropic hormone secretion of the anterior hypophysis, since Klein (1939) demonstrated that thyroidectomy lowered, and hyperthyroidism increased, liver D-amino acid oxidase.

2. Succinic Acid Dehydrogenase. Muscle succinic acid dehydrogenase was similarly investigated in two series of animals. Group IA1 (Table 3) shows an increase of 46% in muscle weight on treatment. Compared with changes of +19% in tibial weight, +25% in body weight, -7% in liver weight and +25% in kidney weight, it is apparent that the great bulk of growth was in the musculature, pro-

TABLE 3. MUSCLE SUCCINIC ACID DEHYDROGENASE

Experiment	No. of rats	Average wt. gain in gm.	Average terminal muscle weight in gm.	Average muscle % N.	Per cent increase in muscle wt. by treatment	Average O ₂ uptake in μ l./h		
						Per gm. tissue	Per mg. total N.	Per 100 gm. body wt.
I. Same group as II in Table 2.								
A. Hypophysectomized.								
1. Treated	5	46	0.469		+46	-4860		-1028
2. Controls	7	5	0.321			-5520		-995
B. Normal								
1. Treated	5	23	0.455		+9	-5400		-1160
2. Controls	6	2	0.417			-5880		-1270
II. 1 month old Sprague-Dawley female rats. Diet of Bartlett (1949). All rats hypophysectomized at 1 month. Plateaued 10 days. Injected with 0.05 mg. growth hormone (1361 R.U./gm.) /rat/day for 6 days, then 0.1 mg./rat/day for 5 days and sacrificed.								
A. Treated	10	14.1	0.174	3.54	+25	-8400*	-237	
B. Controls	10	0.1	0.139	3.45		-8100*	-235	

* Pooled muscle samples assayed.

vided that results for one muscle can be extrapolated to include all muscle. Calculations based on this assumption indicate that 80% of the growth occurred in the muscle, a result in agreement with that of other investigators (Lee and Schaffer, 1934). With this in mind, it is extremely interesting to note that the enzyme concentration was maintained at or near normal values. Thus, 46% of the enzyme present was synthesized as a direct result of growth hormone treatment. In this respect, normal conditions for aerobic oxidation are maintained in this rapidly formed muscle. In view of the known glycostatic effect of growth hormone (Wilhelmi, Fishman, and Russell, 1948), this fact assumes added importance.

A lower level of growth hormone treatment (Table 3, group II) gave increases of 22% in tibial weight, 25% in muscle weight, and 15% in body weight. The muscle response is again about twice that of the total weight gain, but the tibial weight response is even higher than in the first experiment, in which the treatment period was longer and amount of growth hormone given was larger. There are various possible explanations for this difference. The ages were

different, different hormone preparations were used, and tibial weight might have a higher initial growth rate plateauing sooner.

SUMMARY

The effect of growth hormone on kidney and liver D-amino acid oxidase, muscle succinic acid dehydrogenase, and kidney, liver and muscle wet weight was investigated. The concentrations of the two enzymes in their respective tissues remained constant in spite of extensive tissue growth, indicating synthesis of these enzymes in direct proportion to tissue growth. Hypophysectomy resulted in reduced liver D-amino acid oxidase concentrations, probably as a result of reduced thyroid activity.

ACKNOWLEDGMENTS

We wish to express our thanks to Dr. P. D. Bartlett for tissues assayed in Table 3, group II. The growth hormone preparations used in this study were obtained from Parke, Davis & Co. in generous supply through the kindness of Dr. D. A. McGinty and Mr. L. W. Donaldson. The assays had been made by the "plateaued" female rat technique.

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THE EFFECTS OF PITRESSIN AND DESOXYCORTICOSTERONE IN LOW DOSAGE ON THE EXCRETION OF SODIUM, POTASSIUM, AND WATER BY THE NORMAL DOG¹

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IN 1938, Silvette and Britton presented evidence that there exists a physiological antagonism between the diuretic action of adrenal cortical hormone and the antidiuretic action of posterior pituitary hormone. An antagonism between the sodium and chloride conserving effects of the former and the chloruretic and natriuretic effects of the latter has also been postulated—first by Silvette and Britton (1938), and more recently by Roemmelt, Sartorius and Pitts (1949). It has been suggested that the major disturbances in electrolyte and water metabolism of the adrenalectomized animal resemble an exaggerated effect of posterior pituitary antidiuretic hormone (Roemmelt *et al.*, 1949, Birnie *et al.*, 1949). As desoxycorticosterone is known to correct the disturbed electrolyte balance in Addison's disease without significantly altering the disturbed water balance (Talbot *et al.*, 1942; Reforzo-Membrives *et al.*, 1945), it seemed reasonable to predict that desoxycorticosterone would reduce the natriuresis but have no effect on the antidiuresis produced by the administration of pitressin to a normal animal. The data presented in this paper bear out these inferences.

METHODS

Two types of experiments were performed on normal unanesthetized female mongrel dogs weighing from 12 to 18 kilos. In the first type of experiment the dogs were infused with creatinine in 0.45 per cent saline at a rate of 1 cc. per min. in order to determine glomerular filtration rates and the time courses of action of pitressin and desoxycorticosterone. In a second type of experiment the dogs were given a 40 cc. per kilo water load following two 15 minute control periods. In this type of experiment changes in urine flow and sodium excretion were of greater magnitude and more reproducible. We have therefore employed the water loading procedure in an evaluation of the effects of small doses of desoxycorticosterone and pitressin. In order to obtain uniform results it was necessary to standardize procedures in such a

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way as to ensure control urine flows within limits of .25 and .75 cc's per minute and control rates of sodium excretion within limits of 10 and 30 micro-equivalents per minute. Crystalline desoxycorticosterone acetate² dissolved in sesame oil and homogenized with water was given intravenously at the same time as the water load. Due to the delayed action of desoxycorticosterone, pitressin was given intravenously 15 minutes later. Sodium and potassium were determined by an internal standard flame photometer with an accuracy within $\pm 2\%$. Creatinine determination and other techniques have been described in a previous communication (Sartorius *et al.*, 1949).

EFFECT OF INTRAVENOUS PITRESSIN ON RENAL FUNCTION

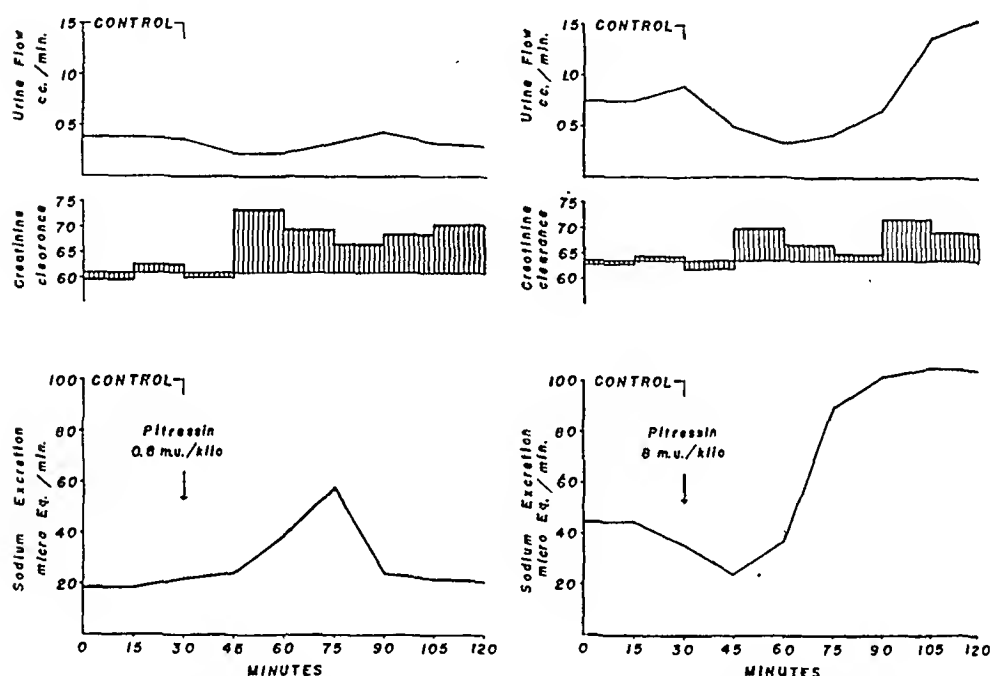


FIG. 1. Immediate effects of intravenous administration of 0.8 and 8.0 m.u. per kilo of pitressin on urine flow, filtration rate and sodium excretion of the normal dog.

RESULTS

Effects of Intravenous Pitressin on Renal Function

In a series of experiments performed on normal, well hydrated dogs, the antidiuretic and natriuretic effects of single intravenous injections of pitressin in dosage from 0.4 m.u. per kilo to 10 m.u. per kilo were determined. Two representative experiments performed on a single animal in which 0.8 m.u. and 8.0 m.u. per kilo of pitressin were employed are shown in figure 1. On the left side of the figure are shown the effects of 0.8 m.u. per kilo of pitressin on urine flow, glomerular

² We are indebted to Ciba Pharmaceutical Products, Inc. for the generous supply of desoxycorticosterone used in these experiments.

filtration rate and sodium excretion, while on the right side the effects of 10 times the dose is charted. In each experiment two 15 minute control periods preceded the pitressin. Following the injection of pitressin no significant change occurred in glomerular filtration rate for the first 15 minutes. However, in the subsequent 15 minute period filtration rate rose sharply in all experiments and then either gradually decreased or remained essentially constant for the remainder of the experiment. No direct correlation was found between the dosage of

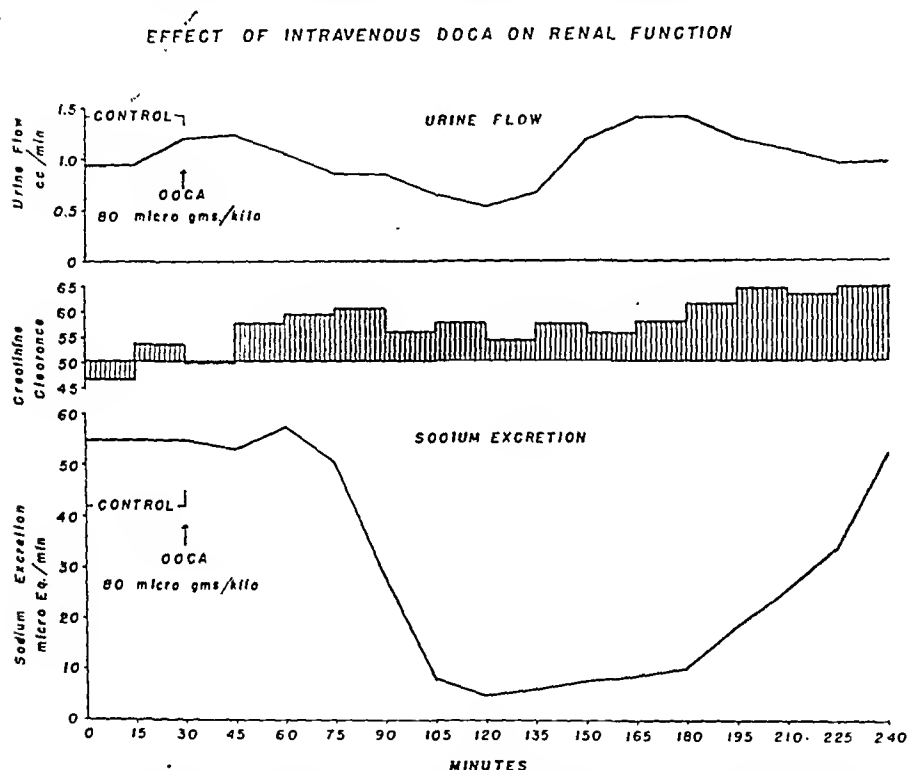


FIG. 2. Immediate effects of intravenous administration of 80 microgms./kilo of desoxycorticosterone on urine flow, filtration rate and sodium excretion of the normal dog.

pitressin and the rise in creatinine clearance. Urine flow invariably decreased immediately following the injection of pitressin reaching a minimum approximately 30 minutes later. Sodium excretion remained unchanged for 15 minutes following the injection. With 0.8 m.u. per kilo, a rise in sodium excretion occurred during the next 30 minutes so that peak excretion was attained approximately 45 minutes following the pitressin. A rapid fall was then commonly observed. As may be seen from the right side of figure 1 when larger doses of pitressin were given the natriuretic effect was more sustained and of greater magnitude although the same latency was observed. It is

interesting to note that with larger dosage of pitressin an increase in urine flow commonly occurred one to $1\frac{1}{2}$ hours after pitressin, on occasion reaching 4-5 cc. per minute. The natriuresis always preceded a pitressin diuresis in these experiments.

Effects of Intravenous Desoxycorticosterone Acetate on Renal Function

The effects of intravenous desoxycorticosterone on renal function in a representative experiment are shown in figure 2. Following two 15 minute control periods, 80 micrograms per kilo of desoxycorticosterone in 1 cc. volume were administered intravenously. As with pitressin a fairly sharp rise in glomerular filtration rate was observed,

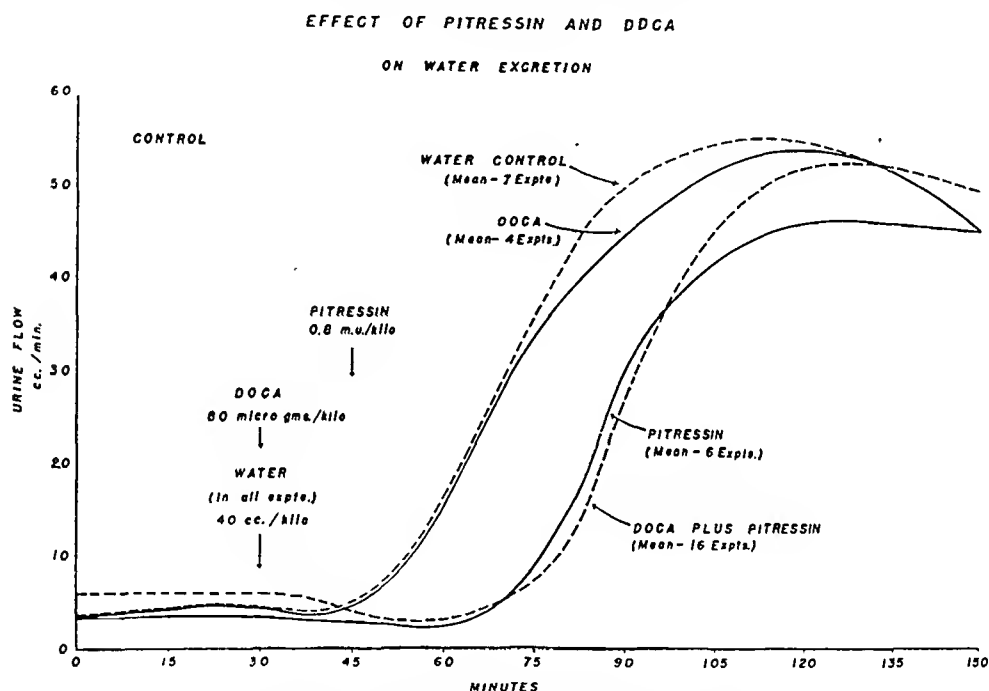


FIG. 3. The effect of pitressin and desoxycorticosterone on excretion of a standard water load by the normal dog.

this elevation being maintained throughout the 4 hour period of the experiment. Urine flow decreased slightly, reaching a minimum at the time of maximum sodium reabsorption. Following the period of maximum sodium reabsorption, there usually occurred a moderate diuresis, often resulting in a total net fluid loss. In the lower half of the chart the effects of desoxycorticosterone on sodium excretion are plotted as a function of time. Following a latent period of approximately 45 minutes sodium excretion decreased rapidly, from an initial level of 65 microequiv. per minute to a low of 5 microequiv. per minute. Diminished sodium excretion was maintained for approximately 75 minutes following which there occurred a gradual return to the control level.

Combined Effects of Pitressin and Desoxycorticosterone on Water Excretion

The data presented in figure 3 provide the basis for our conclusion that desoxycorticosterone has no effect on the elimination of water following a water load nor does it antagonize the antidiuretic action of pitressin. The several curves plotted on this figure are averages of the numbers of experiments noted. For a better evaluation of the combined effects of small doses of desoxycorticosterone and pitressin on water excretion, we have employed the water loading procedure described above. In all experiments therefore, a standard

EFFECT OF WATER, DOCA, AND PITRESSIN ON SODIUM EXCRETION

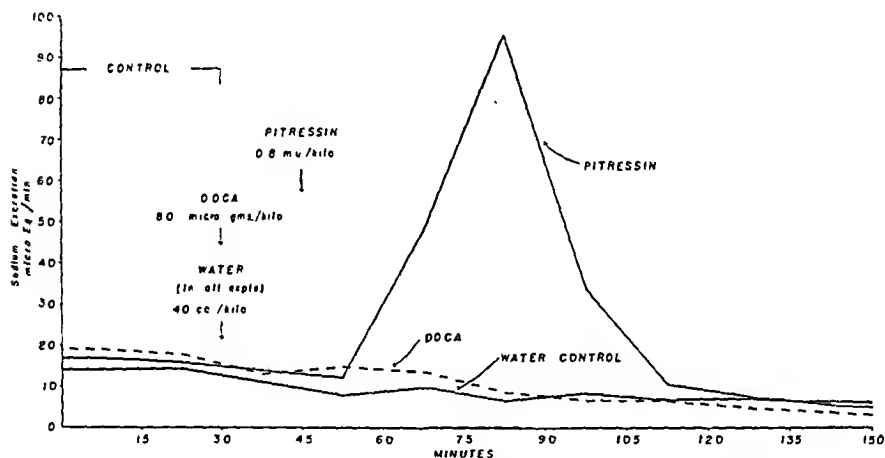


FIG. 4. The effect of pitressin and desoxycorticosterone on sodium excretion by the normal dog under the influence of a standard water load.

water load of 40 cc/kg was administered per os following two 15 minute control periods. In the dashed water control curve no other variable was introduced. This curve therefore constitutes a control for all other procedures. The solid curve adjacent to it and labeled DOCA, differs only in the fact that 80 micrograms per kilo of desoxycorticosterone or 1 to 1½ mg. as a total dose were administered at the same time as the water load. Differences between these curves have no significance. The continuous line labeled pitressin represents the mean urine flow of dogs receiving 0.8 m.u. per kilo of pitressin given 15 minutes after the water load. A marked reduction of output during the subsequent 45 minutes is apparent. At 45 minutes after the water load the average urine flow of the dogs receiving pitressin was 0.9 cc. per minute as compared to 3.6 cc. per minute for the controls. The lower interrupted line labeled DOCA plus pitressin represents the mean urine flow of the dogs receiving the same dose of pitressin but

in addition desoxycorticosterone in dosage varying from 0.8 micrograms per kilo to 80 micrograms per kilo. Again it is evident that desoxycorticosterone exerted no significant effect on water excretion, that is, it does not antagonize the antidiuresis produced by pitressin.

Combined Effects of Pitressin and Desoxycorticosterone on Sodium Excretion

Figure 4 portrays the natriuretic action of pitressin in the water loading experiments just outlined and compares this action to the sodium conserving effect of desoxycorticosterone and of even the water load by itself. In the lowermost curve labeled water control it is apparent that a water load of 40 cc. per kilo, uncomplicated by other procedures diminished sodium excretion gradually from an

EFFECT OF DOCA ON PITRESSIN NATRIURESIS

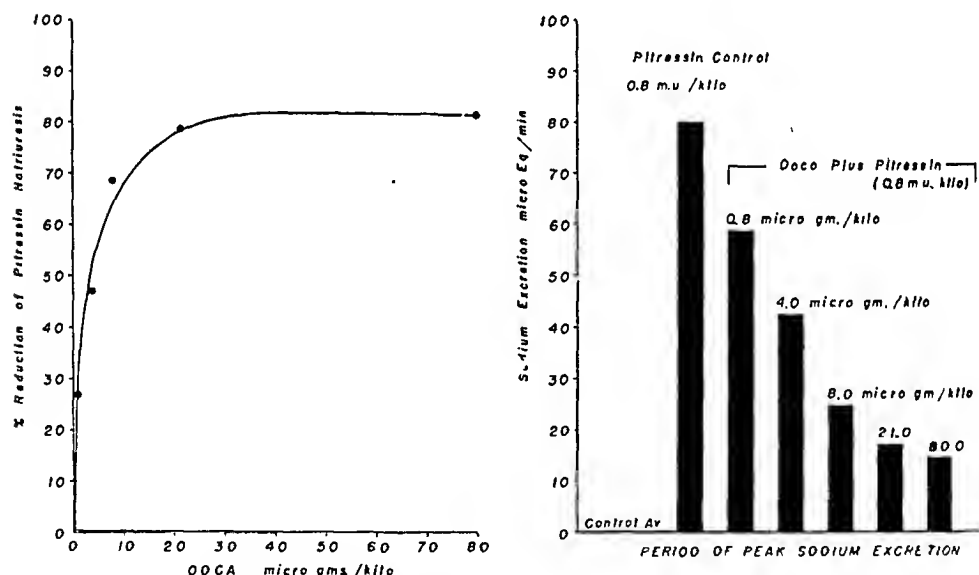


FIG. 5. The antagonistic action of desoxycorticosterone on increased sodium excretion produced by the intravenous administration of 0.8 m.u. per kilo of pitressin to the normal dog.

initial level of 14 microequiv. per min. to 6 microequiv. per min. two hours later. In other experiments of longer duration it has been observed that sodium excretion returned to the control level approximately 3 hours following the water load. Charted as an interrupted line, desoxycorticosterone in dosage of 80 micrograms per kilo reduced sodium excretion from a control level of 18 microequiv. per min. to a low of 3 microequiv. per min. two hours later. Pitressin in dosage of 0.8 m.u. per kilo or 10 to 15 m.u. per dog caused an increased sodium excretion of 80 microequiv. per min. attaining a peak excretion 45 minutes following the injection. There was a latent period of 15

minutes before any natriuretic effect was apparent. Seventy-five minutes following the injection of pitressin sodium excretion again returned to control or sub-control levels.

The effects of desoxycorticosterone on pitressin natriuresis are presented in figure 5. On the right side of the chart, sodium excretion in microequiv. per min. above a control level is shown for the period of peak pitressin natriuresis. The first column represents the mean natriuretic effect of 6 experiments in which 0.8 m.u. per kilo of pitressin was given 15 minutes after the water load. On an average, this dose of pitressin caused the excretion of an extra 80 microequivalents of sodium during its peak period of action. In each of the other 5 columns this same amount of pitressin was given but in addition desoxycorticosterone was administered in the dosage indicated. When 0.8 microgms/kilo of desoxycorticosterone was given, sodium excretion rose to 59 microequiv. per min., a reduction of 21 microequivalents when compared to pitressin alone. Increasing dosage of desoxycorticosterone decreased pitressin natriuresis progressively in stepwise fashion until in the last column when 80 microgms/kilo were given, sodium excretion rose only 14 microequiv. per min. above the base control level.

When these results are plotted as per cent reduction in pitressin natriuresis against desoxycorticosterone dosage the curve shown on the left hand side of figure 5 is obtained. It would thus appear that desoxycorticosterone in any dosage is unable to counteract completely the natriuretic effect of 0.8 m.u. per kilo of pitressin. From other experiments which have not as yet been completed in their entirety it would appear that with smaller doses of pitressin this limitation is negligible, while with larger doses it becomes even more apparent.

Combined Effects of Pitressin and Desoxycorticosterone on Potassium Excretion

Our data on potassium excretion, figure 6, is derived from but ten of the 33 experiments reported above, since the number of experiments suitable for a presentation of average values is limited to those with comparable control potassium excretion rates. However, potassium excretion in all experiments, regardless of control values, showed a similar trend but of varying magnitude. In the lowermost curve of figure 6 and labeled water control, it is apparent that a water load of 40 cc./kilo reduced potassium excretion from an initial level of 21 microequiv. per min. to a low of 8 microequiv. per min. Both desoxycorticosterone in dosage of 80 microgms. per kilo and pitressin in dosage of 0.8 m.u. per kilo increased potassium excretion an average of approximately 13 microequiv. per min. A latent period of 15 minutes preceded the increased rate of potassium excretion due to the administration of desoxycorticosterone. No latent period was apparent after pitressin injection. The combined actions of pitressin and

desoxycorticosterone in the dosage indicated above is graphed as a dash-dot line in figure 6. Almost complete summation is evident.

DISCUSSION

The low volume and high salt content of the urine of adrenalectomized animals and of patients with Addison's disease suggest an exaggerated effect of, or an actual increase in the amount of circulat-

EFFECT OF WATER, DOCA, AND PITRESSIN ON POTASSIUM EXCRETION

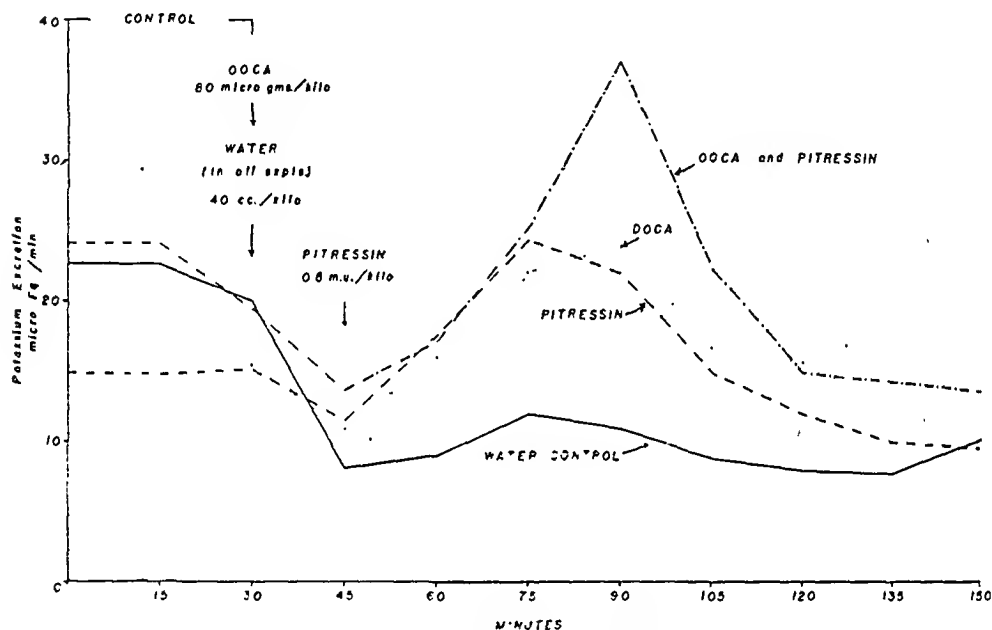


FIG. 6. The synergistic effect of pitressin and desoxycorticosterone administration to the normal dog on potassium excretion.

ing posterior pituitary antidiuretic hormone (Roemmelt *et al.*, 1949, Birnie *et al.*, 1949). This hypothesis recognizes the antidiuretic, chloruretic and natriuretic actions of the posterior pituitary hormone as being in physiological balance with the diuretic and salt conserving actions of the cortical hormones (Silvette and Britton, 1938, Corey, Silvette and Britton, 1939 and Winter *et al.* 1939). Assuming the above concept to be true, it seemed logical that the administration of pitressin in small dosage to a normal animal would produce a mild pseudo-Addisonian state in respect to electrolyte and water balance and that such disturbances would be amenable to desoxycorticosterone administration to the same extent as are those of the adrenalectomized animal.

That pitressin inhibits water diuresis is well known (Smith, 1947). That desoxycorticosterone exhibits diuretic properties has been

suggested by numerous investigators (Selye and Basset, 1940; Corey and Britton, 1941; Eversole *et al.* 1942). As the data in the experiments pertaining to the diuretic activity of desoxycorticosterone were derived from 11, 12 or 24 hour urine collection periods, the *modus operandi* of this action has not been clarified. Ragan *et al.* 1940 investigated the diuretic action of desoxycorticosterone by producing in dogs, a diabetes insipidus like state through the administration of 25 mg. of the drug daily. They arrived at the conclusion as did Mulino, 1941 that this condition was due primarily to increased thirst of the animals and that the polyuria was a secondary manifestation. This concept is in accord with our findings that the intravenous administration of desoxycorticosterone results primarily in increased salt reabsorption which is attended by increased water absorption of lesser magnitude, as shown in figure 2. Any subsequent diuresis then would be secondary to expanded blood volume as a consequence of desoxycorticosterone administration (Swingle and Remington, 1944), to elevation of filtration rate (Winter and Ingrams, 1943), or to increased water intake dependent upon increased sodium reabsorption (Anderson and Marlin, 1942). It has also been shown that the deficiencies in handling water of 6th day post adrenalectomized rats can be only partially corrected by the administration of either desoxycorticosterone or whole cortical extract (Gaunt, 1946). Patients with Addison's disease show a similar refractoriness to correction of water balance (Reforzo-Membrives, 1945, and Talbott, 1942). Since pitressin exerts a specific action on the capacity of the renal tubule to reabsorb water (Smith, 1947) and since desoxycorticosterone has been shown in the above experiments to exert neither a direct action on water reabsorption per se, nor an antagonistic action to pitressin antidiuresis, clarity is added to our understanding of the adrenalectomized animal's relative incapacity to excrete water under desoxycorticosterone therapy. Whether some fraction of the adrenal cortex possesses direct diuretic activity (Silvette and Britton, 1938) or whether the intra-renal circulation undergoes alteration as a result of the exaggerated effects of antidiuretic hormone (Trueta *et al.* 1947) thus accounting for the progressive refractoriness of the adrenalectomized rat's ability to excrete water under therapy (Gaunt, 1946) is not clear at the present time.

The natriuretic action of pitressin has been previously demonstrated (Anslow *et al.*, 1948 and Roemmelt, 1949) and has, in the present experiments, been found to be of fairly constant magnitude and extent. Desoxycorticosterone in low dosage progressively but never completely counteracted the natriuretic activity of 0.8 m.u. per kilo of pitressin as shown in figure 6. It seems probable that with lower pitressin dosage, the antagonistic action of desoxycorticosterone on sodium absorption would be complete. We would also suspect from the above findings that the physiological dose of pitressin lies

somewhat below a level of 0.8 m.u. per kilo. The efficacy of desoxycorticosterone therapy in correcting and maintaining proper sodium excretion in adrenalectomized animals and in Addisonian patients has been adequately demonstrated (Thorn, 1938, Britton and Kline, 1941).

Potassium excretion is diminished in the adrenalectomized animal (Harrop, 1933) and can be increased by the administration of desoxycorticosterone (Wells, 1940 and Swingle, 1944). Our experiments indicate that pitressin in low dosage increases the excretion of potassium. This increase is independent of the hyperkalemia which results from the injection of fairly large doses of pitressin (Yanagi, 1938) and likewise bears no relationship to the increase in filtration rate often observed following the injection of 0.8 m.u. per kilo of pitressin. This observation at first glance does not appear compatible with a concept of adrenal and posterior pituitary hormonal antagonism. However, since the independent actions of pitressin and desoxycorticosterone showed almost complete summation of the excess potassium excreted when used in combination, it becomes apparent that the adrenalectomized animal should, and indeed does, show a diminished rate of potassium excretion. It is likewise evident that the administration of pitressin to a normal animal cannot reproduce in its entirety the Addisonian electrolyte and water imbalances, since the combined effect of pitressin and cortical hormone on potassium excretion is additive rather than antagonistic.

In conclusion, it appears that desoxycorticosterone is antagonistic to the posterior pituitary antidiuretic hormone as to sodium excretion, synergistic as to potassium excretion, and without direct effect as to water excretion.

SUMMARY

The effects of intravenous injection of desoxycorticosterone and pitressin were studied as to filtration rate, sodium excretion and urine flow of the normal dog. The time courses of action of these drugs were determined.

In a second type of experiment, water excretion curves were established for dogs given 40 cc./kilo water load following two 15 minute control periods. Pitressin in dosage of 0.8 m.u. per kilo given 15 minutes following the water load exerted an antidiuretic effect of $1\frac{1}{2}$ to 2 hours duration. Desoxycorticosterone in dosage from 0.8 micrograms per kilo to 80 micrograms per kilo exerted no effect on either water diuresis or on the antidiuretic action of pitressin.

Eight-tenths micrograms per kilo of pitressin caused the excretion of an extra 80 microequiv. per min. of sodium during its peak period of action. Increasing dosage of desoxycorticosterone decreased the pitressin natriuresis but did not completely block this effect.

Both desoxycorticosterone and pitressin independently increased

the rate of potassium excretion and when given in combination showed almost complete summation of the excess potassium excreted.

The interrelationships and antagonistic actions of the posterior pituitary antidiuretic hormone and the adrenal cortical hormones are discussed.

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We wish to thank Dr. R. F. Pitts for his kind interest and helpful guidance in this project.

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FACTORS INFLUENCING LOBULO-ALVEOLAR DEVELOPMENT AND MAMMARY SECRETION IN THE RAT¹

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DURING pregnancy, the mammary gland of the rat undergoes a marked transformation so that, near the end of gestation, it consists of grape-like clusters of alveoli in lobule formation. Two days before parturition, secretion within the alveoli becomes visible (Turner and Schultze, 1931). Full development of the mammary gland, therefore, is attained when lobulo-alveolar development with secretion is established. In the absence of pregnancy, full development of the mammary gland has been produced experimentally in rats by injection of the gonadotropic substance from the urine of pregnant women (Selye *et al.*, 1933). This hormone causes intense luteinization of the ovaries and complete lobulo-alveolar development of the gland but no secretion. If the stimulated ovaries are excised, secretion will follow about thirty-six hours later and the gland will then present the appearance of full development.

It is well recognized that estrogen in physiological doses is only capable of stimulating extension and branching of the ducts of the mammary gland of the rat (Astwood and Geschickter, 1938). Therefore, one might deduce from these findings that progesterone or a combination of estrogen and progesterone is responsible for lobulo-alveolar development in the mammary gland of the rat. Removal of the luteinized ovaries followed by secretion within the alveoli is a noteworthy example of a withdrawal effect. It has been amply demonstrated that estrogen can suppress lactation (Edelmann and Gaunt, 1941) but, also, it has been shown that large doses of estrogen administered to an oophorectomized rat will cause formation of cysts of various sizes arising from the termini of the ducts and filled with secretion (Astwood and Geschickter, 1938). Therefore, the part that estrogen plays in the secretion of the mammary gland is still controversial. It would not be unreasonable to suppose that progesterone withdrawal is responsible for the initiation of lactation just prior

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to parturition in view of experimental evidence which shows that the level of progesterone drops at the end of gestation. Bachman and co-workers (1940) demonstrated a pre-labor drop in pregnanediol excretion, and Lyon (1946) likewise found that the pregnanediol excretion in human beings during the last two weeks of pregnancy declined and that the fall became abrupt during the last six days prior to the onset of labor. Other investigators (Atkinson and Hooker, 1945; Atkinson and Leathem, 1946) have examined the histological changes in the uterus of the mouse from day to day in pregnancy and lactation and they have found that, whereas a progesterone effect was predominant in the middle of pregnancy, the changes at the end of gestation were characteristic of estrogen stimulation.

In 1931, Turner and Schultze injected a corpus luteum extract into ovariectomized rats without any effect on the mammary gland, but combined administration of an estrogen from the urine of pregnant cows and the corpus-luteum extract in spayed female rats produced lobule formation. Astwood and Geschickter (1938) repeated this experiment later with crystalline progesterone alone and together with estrone; the largest dose, 1 mg. of progesterone and 5 μ g. of estrone daily for eight days, induced no demonstrable lobulo-alveolar growth. In 1948, Meites and Turner reported that limited lobulo-alveolar development but no secretion could be obtained in oophorectomized rats after injection of 500 IU of estrone with 15 mg. progesterone every day for ten days. Selye (1940) claimed to have obtained extensive lobular development in the mammary gland of oophorectomized rats by injection of 15 mg. of progesterone alone every day for ten days. Hamolsky and Sparrow (1945) gave various combinations of estradiol, progesterone, and relaxin after pretreatment with estradiol and found that lobulo-alveolar development was more advanced at the end of nine days among spayed rats that received relaxin with estrogen and progesterone than in animals given only estrogen and progesterone. It is easily seen that there is a discrepancy among the findings pertaining to the relative importance of certain reproductive hormones in influencing the mammary gland.

This paper presents results of an investigation of the influence of estrogen and progesterone in bringing about complete lobulo-alveolar development of the mammary gland and mammary secretion in immature, ovariectomized rats.

MATERIAL AND METHODS

The animals used were rats of the Long Evans strain fed on Purina fox checkers. The mammary glands were studied in whole mounts; the partly fixed glands were dissected away from the subcutaneous tissue, fixed in Bouin's solution, stained in aqueous alum hematoxylin, cleared in xylol, and mounted in Canada balsam. Also, paraffin sections of many glands preserved in Bouin's solution and stained with hematoxylin and eosin were

TABLE 1. SUMMARY OF EXPERIMENT WITH CHORIONIC GONADOTROPIN

Daily dose of chorionic gonadotropin (I.U.)	Duration of treatment with chorionic gonadotropin (days)	Day killed
5	4	5
5	7	8
5	11	12
5	14	15
5	17*	19

* Ovariectomized on seventeenth day.

made. The whole mounts were most useful in viewing the gross architecture of the gland but, for actual detail, such as evidence of secretion, the hematoxylin and eosin sections proved to be more reliable. The endocrine preparations used were chorionic gonadotropin² from human pregnancy urine, estradiol benzoate³ in sesame oil, aqueous suspensions of progesterone,⁴ and progesterone⁵ in sesame oil. No injections were given on Sundays and all injections were subcutaneous. The investigation was divided into four parts.

One group of immature, female rats was given chorionic gonadotropin according to the schedule outlined in Table 1. The animals were killed at intervals of several days after the start of injections so that the mammary glands could be removed for examination. It is to be noted that the ovaries

TABLE 2. SCHEDULE OF TREATMENT WITH ESTRADIOL AND PROGESTERONE

Daily dose of aqueous suspension of progesterone (mg.)	Duration of treatment with progesterone (days)	Dose of estradiol twice a week (mg.)	No. of estradiol injections	Day killed
—	—	0.1	6	22
—	—	0.001	6	22
4	21	—	—	22
15 (in oil)	12	—	—	13
4	21	0.1	6	22
4	21	0.001	6	22

were excised on the seventeenth day and the mammary glands were removed two days later. The second group of immature, female rats were oophorectomized and then given estradiol benzoate twice a week and progesterone daily, singly and together in various doses, for twenty-one days as indicated in Table 2. The third group of immature, female rats were also oophorectomized and then given a combination of estradiol benzoate twice a week and progesterone daily for seventeen days. No more progesterone was given after the seventeenth day and the last estradiol injection was administered on the eighteenth day. This experiment is summarized in Table 3. Rats were killed and glands were removed on each of five successive days after cessation of progesterone injections. The fourth group of animals was composed of nor-

² "Korotrin," Winthrop Chemical Company.

³ "Progynon B," Schering Corporation.

⁴ Parke, Davis, and Company.

⁵ "Proluton," Schering Corporation.

TABLE 3. SCHEDULE OF TREATMENT WITH ESTRADIOL AND PROGESTERONE FOLLOWED BY PROGESTERONE WITHDRAWAL ON THE EIGHTEENTH DAY

Daily dose of progesterone in oil (mg.)	Duration of treatment with progesterone (days)	Dose of estradiol twice a week (mg.)	No. of estradiol injections	Day killed	Day of progesterone withdrawal on day killed
5	17	0.166	5	18	1
5	17	0.166	6	19	2
5	17	0.166	6	20	3
5	17	0.166	6	21	4
4	17	0.100	6	21	4
(in aqueous suspension)					
5	17	0.166	6	22	5

mal female pregnant rats whose mammary glands were removed on the eleventh, seventeenth, eighteenth, nineteenth, twentieth, and twenty-first day of gestation and first day post-partum after suckling had begun.

RESULTS

Part I. Effect of Chorionic Gonadotropin. Daily injections of 5 IU chorionic gonadotropin into immature rats resulted in marked ovarian enlargement from the formation of numerous corpora lutea. The vagina in all cases opened by the fifth day of treatment and all smears were characteristic of estrus at this time. Subsequently, diestrus spreads for the most part were obtained. A gland on the fifth day after treatment simply showed extension of the duct tree and increased branching typical of an estrogen effect. On the eighth day after treatment the gland presented a different appearance entirely. At the termini of the ducts clusters of alveoli had formed. On the twelfth day after injection, larger clusters of alveoli were seen. The alveoli were packed closely together, thus giving the whole gland a denser appearance (Fig. 1). Lobule formation of these alveolar clumps was definite. The mammary gland on the fifteenth day after treatment showed similar changes. There was no gross evidence of secretion in any of these glands and no significant difference in size. A mammary gland on the nineteenth day two days after oophorectomy revealed doubling in size over the other glands and marked distention of alveoli with secretion (Fig. 2). It had not been possible to express milk from the gland prior to death.

Part II. Effect of Estradiol and Progesterone on Immature Oophorectomized Rats. 0.1 milligram of estradiol benzoate, given twice a week for three weeks, resulted in glands composed of cysts of all sizes containing secretion, irregular dilatation of the ducts, and stunted growth (Fig. 3). One-thousandth milligram of estradiol benzoate, administered in the same manner, induced extensive growth, arborization of ducts, and beginning cyst formation (Fig. 4). The administra-

tion of 4 mg. of progesterone daily for twenty-one days caused no detectable change in the mammary gland. A larger dose of 15 mg. daily for twelve days also appeared to have no effect (Fig. 5). It is interesting to contrast the appearance of the gland of a rat that was kept separately while receiving 4 mg. of progesterone daily for twenty-one days with one of a rat that, while being given the same dose of progesterone, was placed in a cage with animals being given estradiol. In the gland of the rat placed with other animals getting estradiol, incomplete lobulo-alveolar development occurred (Fig. 6). There was no change in the gland of the rat that was kept apart while getting progesterone. It is supposed that estradiol was obtained from the other animals that were receiving this substance. Combined treatment with estradiol benzoate twice a week, and 4 mg. of progesterone daily over a period of twenty-one days, resulted in complete lobulo-alveolar development but no secretion (Fig. 7). The gland of the rat that was given 0.1 mg. of estradiol benzoate with 4 mg. of progesterone proved to have more solidly packed alveoli than the gland of the rat that received only 0.001 mg. of the estrogen with 4 mg. of progesterone.

Part III. Effect of Progesterone Withdrawal Following Estradiol and Progesterone Administration in Immature Oophorectomized Rats

The withdrawal of progesterone on the eighteenth day after attaining complete lobulo-alveolar development with estradiol and progesterone was followed on the twentieth day, or the third day of withdrawal, by beginning secretion within the alveoli. Secretion reached a maximum on the twenty-first day, or the fourth day of withdrawal, and milk could be expressed through the nipple. Secretion began to recede on the twenty-second day. A better secretory response and more prominent nipples were obtained in those rats that received with the estradiol the aqueous suspension of progesterone rather than progesterone in oil. On the eighteenth day, or first day of withdrawal, clumps of closely packed alveoli were found (Fig. 8). Each alveolus was composed of a ring of high cuboidal epithelium with granular cytoplasm without secretion in the lumen. There was a moderate amount of fat tissue supporting the epithelial structures. Except for a slight increase of fat around the gland, the gland excised on the nineteenth day did not differ from the one on the eighteenth day. On the twentieth day, or third day of withdrawal, there was marked increase in the density of the gland, slight increase in size and more fat in the glandular region greatest around the base of the gland and least in the region of the nipple. Microscopically, there was beginning secretion within the alveoli, and there were numerous small fat globules within the cytoplasm of the epithelial cells, as well as in the material secreted into the lumen (Fig. 9). By the twenty-first day, there was even more secretion which was widespread throughout the gland, and milk could be expressed through the nipple. At the same time, the epithelial cells had flattened suggesting a massive discharge of material

which they had previously been storing. Fat globules were again seen in the cytoplasm and in the material secreted (Figs. 10 and 11). As will be seen later, this gland was similar in microscopic appearance to the mammary gland on the twenty-first day of pregnancy just before parturition. On the twenty-second day, the gland was less thick and secretion had begun to recede.

Part IV. Mammary Gland Changes During the Last Half of Pregnancy. By the eleventh day of gestation, alveoli in small clusters were beginning to form in the central portion of the gland while ducts only with their small end buds still occupied the periphery. Hematoxylin and eosin sections showed collapsed ducts, a few small groups of alveoli, and abundant fat tissue. Glands removed on the seventeenth day showed a greater profusion of alveolar clumps supported in fatty tissue. Hematoxylin and eosin sections showed no evidence of secretion. Excision of the gland on the eighteenth day revealed incomplete lobulo-alveolar development. There was a rare alveolus filled with secretion, but, for the most part, no secretory changes had taken place. Glands removed on the nineteenth and twentieth days showed pronounced lobulo-alveolar development, alveoli composed of cuboidal epithelium with markedly vesicular cytoplasm, and very slight secretion in some areas. By the twenty-first day, widespread secretion had taken place and there was marked reduction in height of the epithelium to a low cuboidal state (Fig. 12). Small fat globules were present in the cytoplasm as well as in the material secreted. Milk could not be expressed at this time. These changes were similar to those found in the glands of oophorectomized rats that had been given estradiol and progesterone followed by progesterone withdrawal after lobulo-alveolar development had been attained (Fig. 10). One day post-partum, after suckling had begun, there was marked distention of the alveoli with very little supporting fatty tissue seen. The alveolar epithelium was very flat and apparently broken in places so that a number of the lumina of the alveoli were connected.

DISCUSSION

There is no longer any uncertainty that the mammary gland is under hormonal control, but there is still much to be learned about the interaction of the specific hormones involved. It is felt that the experiments performed have shown that lobulo-alveolar development as extensive as that seen in normal pregnancy in the rat mammary gland can be produced by estrogen and progesterone. To date, this appears to be the greatest amount of lobulo-alveolar development obtained in the spayed rat with these hormones. The most advanced changes were obtained with 0.1 mg. of estradiol benzoate with 4 mg. of progesterone. This dose of estrogen may be excessive under normal circumstances but, during pregnancy, this amount might well lie within the physiological range.

That prolactin plays an important role in lactation appears to have been well established (Meites and Turner, 1948). However, the manner in which its action is coordinated with the ovarian hormones has been largely a matter of conjecture. It has been shown that estrogen increases the concentration of prolactin in the anterior pituitary (Meites and Turner, 1948). It is well established, too, that prolactin can stimulate secretion in the mammary gland, provided the latter is properly developed (Turner, 1948). The secretory action of prolactin is probably a direct one as demonstrated by Lyons (1942), who by injecting prolactin into single milk ducts obtained local sector lactation in the rabbit's mammary gland, which had been developed by estrogen and progesterone. In addition to this function, it has been shown that Prolactin causes luteal tissue to secrete progesterin (Evans *et al.*, 1941), and in all probability, prolactin and the hypophyseal luteotrophin described by Astwood (1941) are identical. Stimulation of luteal tissue by prolactin to form progesterone, as well as production of mammary gland secretion by prolactin, are difficult to reconcile in view of the experimental evidence of inhibition of secretion by progesterone. However, these two effects of prolactin may be resolved if one examines the sequence of events in normal pregnancy. During the first half of pregnancy in the rat, corpus luteum function is maintained by the anterior pituitary, probably through the secretion of prolactin, but no mammary secretion occurs. This may either be a result of interference by progesterone or because the mammary gland has not reached the proper stage of development to be stimulated in this manner. During the last half of gestation in the rat, the placenta assumes the work of stimulating the function of the corpus luteum by secreting a chorionic luteotrophin which thus enables continued progesterone formation (Astwood and Greep, 1938). Apparently, near term, placental function and therefore progesterone formation decreases. In the human being, during the last two-thirds of pregnancy, the placenta secretes estrogen and progesterone. As a result of progesterone withdrawal prior to parturition, prolactin,

FIG. 1. (top) Mammary gland of an immature female rat treated for twelve days with 5 IU chorionic gonadotropin. $\times 25$.

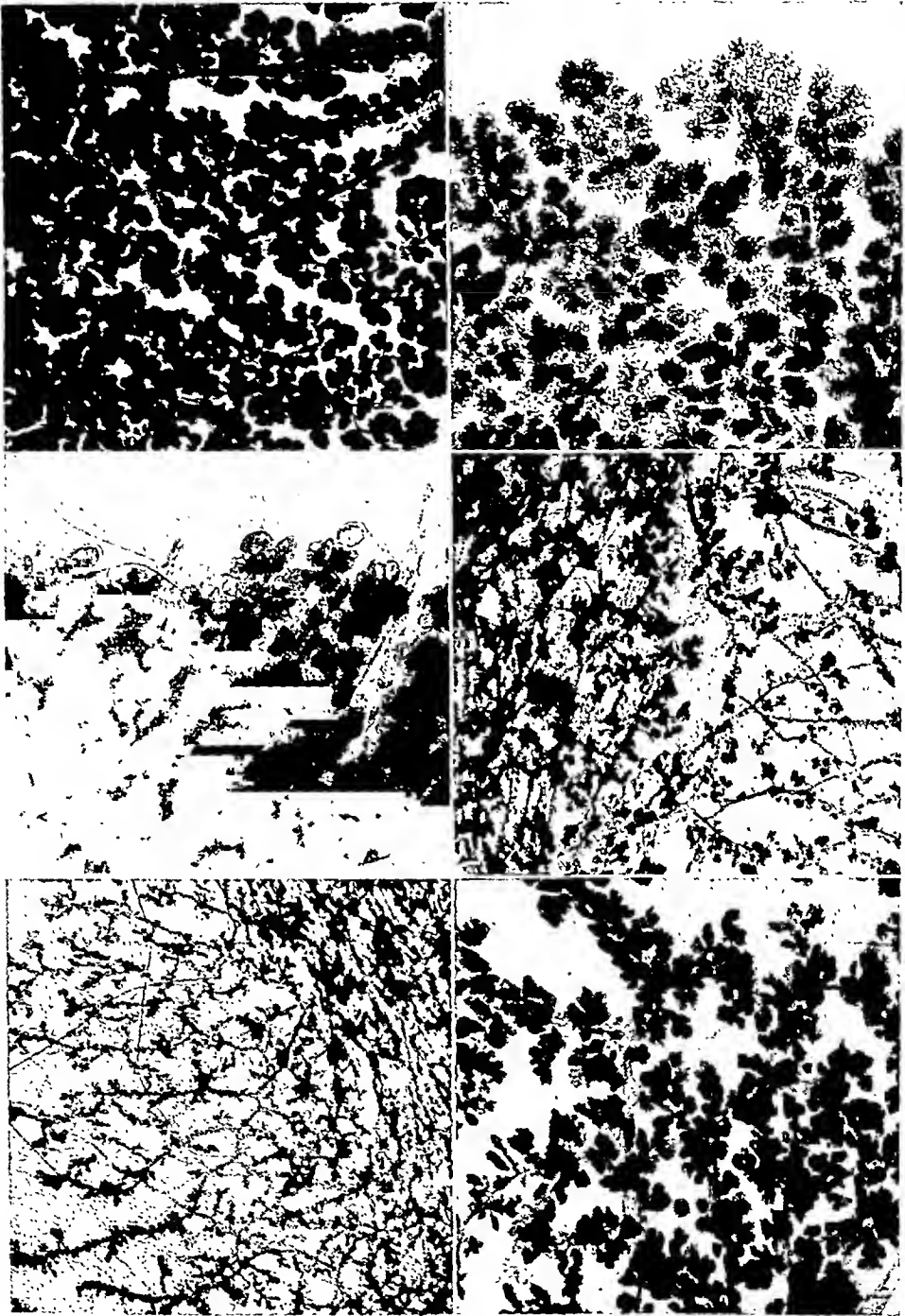
FIG. 2. (top right) Mammary gland of an immature female rat treated for seventeen days with 5 IU chorionic gonadotropin, oophorectomized on the seventeenth day and killed two days later. $\times 25$.

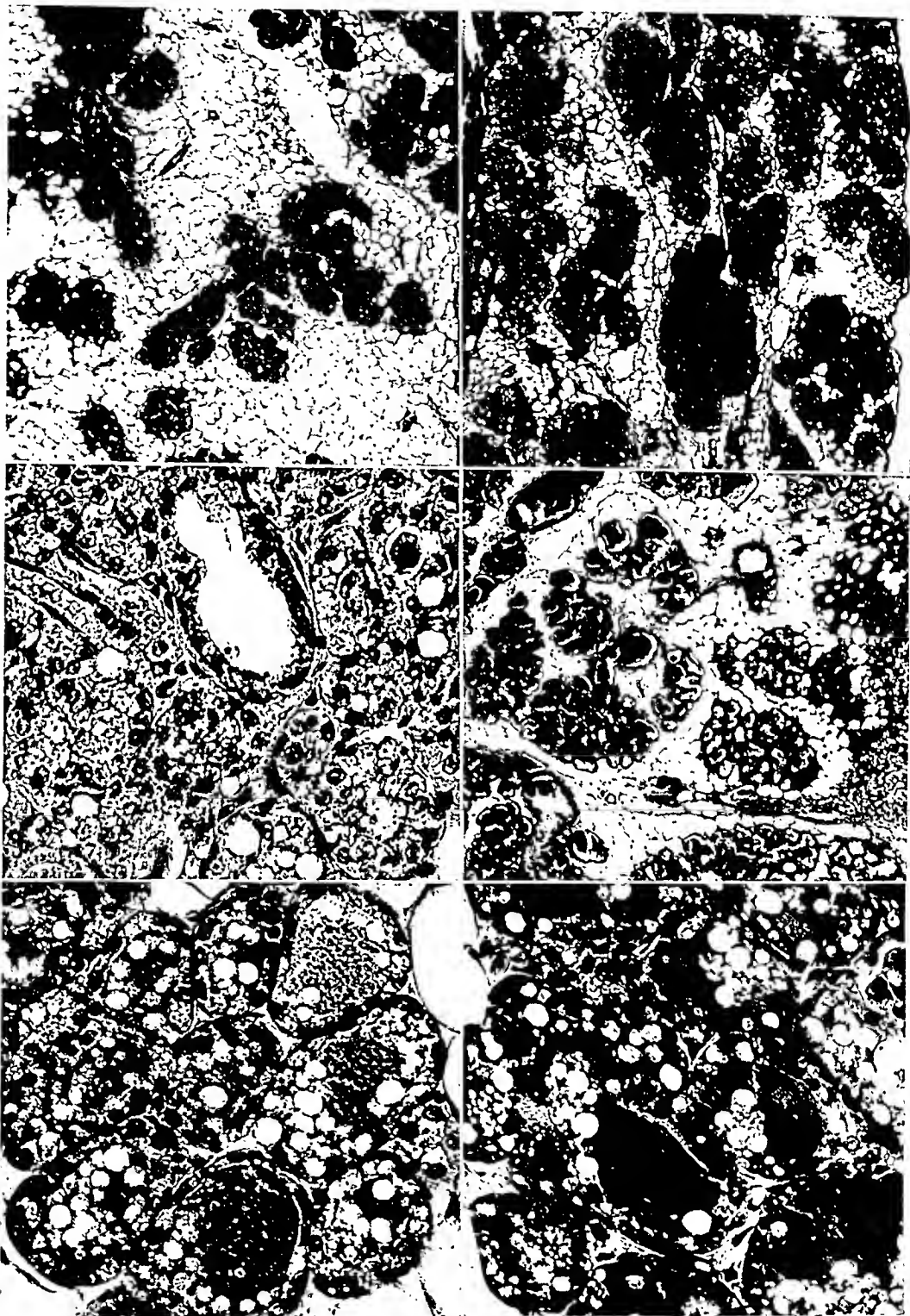
FIG. 3. (center left) Mammary gland of an immature spayed female rat treated twice a week for three weeks with 0.1 mg. estradiol benzoate. $\times 25$.

FIG. 4. (center right) Mammary gland of an immature spayed female rat treated twice a week for three weeks with 0.001 mg. estradiol benzoate. $\times 25$.

FIG. 5. (bottom left) Mammary gland of an immature spayed female rat treated daily for twelve days with 15 mg. progesterone in oil. Animal was kept in a separate cage while receiving progesterone. $\times 25$.

FIG. 6. (bottom right) Mammary gland of an immature spayed female rat treated daily for twenty-one days with 4 mg. progesterone in aqueous suspension. While getting progesterone, rat was kept in a cage with other animals being given estradiol. $\times 25$.





perhaps stimulated by estrogen, is then able to exert its secretory effect upon the mammary gland. It would appear, therefore, that secretion in the mammary gland is dependent upon the proper balance between prolactin and progesterone. By raising the prolactin to progesterone ratio, either by increasing prolactin or by decreasing progesterone, one, therefore, might induce secretion. In pregnancy, suppression of secretion is probably due to the high concentration of circulating progesterone. As a result of its abrupt fall at the end of gestation, prolactin is then able to evoke a secretory response in the gland.

It has been ascertained that estrogen requires the anterior hypophysis in bringing about growth and secretion (Leonard and Reece, 1942). The type of growth that estrogen induces in intact animals varies with the species. In the cow, goat, and guinea-pig, complete lobulo-alveolar development follows estrogen administration alone, while in the rat, mouse, and rabbit, only duct growth occurs, progesterone being necessary in addition for lobulo-alveolar growth. The nature of the pituitary factor essential for mammary growth is not known, but in all probability it is different from prolactin (Greep and Staveland, 1941).

Progesterone is essential for the maintenance of pregnancy in all mammals but it is not needed, as mentioned above, for lobulo-alveolar development of the mammary gland in all species. However, the presence of progesterone in high concentrations during pregnancy seems to be the prime factor in inhibiting lactation in mammals. The mechanism of this inhibition is not known, but it is entirely possible that it acts locally on the epithelial cells. In the rat, progesterone not only may prevent discharge of cellular material, but it also acts in conjunction with estrogen in promoting lobulo-alveolar growth. In view of the fact that prolactin also seems to act locally, it might act to release materials from the cytoplasm previously held in check by progesterone.

FIG. 7. (top) Mammary gland of an immature spayed female rat treated with 0.1 mg. estradiol twice a week and 4 mg. progesterone in aqueous suspension over a period of twenty-one days. Unfortunately, the photograph taken of the section was not representative of the entire field which included a greater number of clusters of alveoli. $\times 100$.

FIG. 8. (top right) Mammary gland of an immature spayed female rat on first day of withdrawal of progesterone after attaining lobulo-alveolar development with estrogen and progesterone. $\times 100$.

FIG. 9 (center left) Mammary gland of an immature spayed female rat on third day of progesterone withdrawal after attaining lobulo-alveolar development with estrogen and progesterone. $\times 800$.

FIG. 10 (center right) Mammary gland of an immature spayed female rat on fourth day of progesterone withdrawal after attaining lobulo-alveolar development with estrogen and progesterone. $\times 100$.

FIG. 11. (bottom left) Same as Figure 10. $\times 800$.

FIG. 12. (bottom right) Mammary gland of a normal pregnant rat on the twenty-first day of gestation. $\times 800$.

Experiments on hormonal inhibition of established lactation have yielded variable results. Hypophysectomy in the rat during the second half of gestation does not prevent the onset of lactation just after parturition (Selye *et al.*, 1933). However, this secretion lasts only a few hours. No satisfactory explanation of this phenomenon has been presented. Hypophysectomy stops the flow of milk promptly when the operation is performed at any time after delivery (Selye *et al.*, 1933). It has been reported that estrogen can inhibit lactation in oophorectomized rats, but it is more effective in intact rats (Edelmann and Gaunt, 1941). Gonadotropins, likewise, (Edelmann and Gaunt, 1941) mediate their lactation-inhibiting properties through the ovary, which suggests that both hormones function by virtue of a direct or indirect luteotrophic action. Thus far, progesterone administration has usually failed to inhibit milk flow (Edelmann and Gaunt, 1941). The flow of milk post-partum arises as a result of local stimulation of the nipple by suckling, and this may in part be mediated by an increased elaboration of prolactin. In view of the probable individual variation in response to suckling, one should not be surprised to obtain variable degrees of inhibition by hormones. The response of lactating human beings to estrogen is most inconsistent (Birnberg *et al.*, 1947). Sometimes no effect has been observed. In others only a relief of congestion has been experienced, and, in a few, a decrease of flow has been obtained. In the last analysis it would appear that the predominant mammary effect during gestation until just prior to parturition is that of progesterone which, while furthering lobulo-alveolar development in most species, interferes with secretory action of prolactin. The hormonal effect predominating during the suckling period is prolactin, which may be able to override any effect by progesterone or substances such as estrogen or gonadotrophin that simulate the secretion of progesterone.

SUMMARY

Secretion in the mammary gland of the normal pregnant rat appears about two days prior to parturition. Chorionic gonadotropin causes intense luteinization of the ovaries and complete lobulo-alveolar development of the mammary gland in the intact rat but no secretion. If the luteinized ovaries of rats treated with chorionic gonadotropin are excised, secretion in the developed mammary gland will follow in forty-eight hours. Estrogen and progesterone when given together to ovariectomized immature rats in suitable doses cause complete lobulo-alveolar development of the mammary gland but no secretion. Withdrawal of progesterone after obtaining lobulo-alveolar development with estrogen and progesterone in ovariectomized immature rats results in mammary gland secretion which is greatest on the fourth day of withdrawal. The occurrence of secretion appears to

be dependent upon the ratio of prolactin to progesterone acting upon the mammary gland.

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THE PARADOXICAL EFFECTS OF THIOCYANATE AND OF THYROTROPIN ON THE ORGANIC BINDING OF IODINE BY THE THYROID IN THE PRESENCE OF LARGE AMOUNTS OF IODIDE

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IT HAS been demonstrated by Wolff and Chaikoff (1948) that the administration of a large dose of iodide to the intact rat inhibits the organic binding of iodine by the thyroid gland. In the experiments reported, the inhibition was correlated with the plasma iodide concentration, and the effective values were found to be above 20–35 $\mu\text{g. per } 100 \text{ cc.}$

The ability of thiocyanate and of thyrotropin to affect the iodide concentrating power of the thyroid offered a means for manipulating the iodide content of the gland without changing the plasma iodide concentration appreciably. Thiocyanate markedly inhibits the capacity of the thyroid to concentrate iodide ion (Franklin, Chaikoff, and Lerner, 1944; VanderLaan and VanderLaan, 1947), and, at physiological plasma iodide concentrations, slows the process of synthesis of organic iodine compounds (Wolff, Chaikoff, Taurog, and Rubin, 1946). Thyrotropin increases the iodide concentrating power (Stanley and Astwood, 1949) and has the effect, at ordinary iodide values, of increasing the rate of organic binding (Leblond and Sue, 1941; Morton, Perlman, and Chaikoff, 1941). Paradoxical effects on the rate of organic synthesis would be expected from the administration of thiocyanate and of thyrotropin in the presence of high plasma iodide concentrations if the iodide inhibition were a function of the iodide concentration in the thyroid gland.

METHODS

Long-Evans rats on a diet of Purina Fox Checkers were used. When pre-treated with propylthiouracil, the drug was given in a 0.03 per cent concentration in Purina Mink Chow. The low-iodine diet used in some experiments was made from the following formula: wheat gluten, 360 Gms.; yellow corn meal, 1400 Gms.; dried brewer's yeast, 100 Gms.; salt, 20 Gms.

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All determinations were made by radioactivity measurements. Iodide, as potassium iodide, was labelled with radioactive iodide (I^{131}) and the specific activity of this solution (cts./sec./ μ g.) determined. The iodide solution was injected intraperitoneally. The amount of iodide injected was always many times greater than the total iodide content of the animal. The latter was therefore neglected in all calculations. Potassium thiocyanate, when used, was given at the same time and by the same route as the iodide. Thyrotropin was injected subcutaneously.

At the termination of the experimental period, the rat was anesthetized with ether, the chest opened, blood obtained for plasma I^{131} content by heart puncture, and the animal killed by exsanguination. The thyroid glands were then removed immediately, weighed, and transferred to glass grinders where they were homogenized in 0.5 cc. saline by grinding between fitted ground glass surfaces. The homogenate was quickly transferred to a centrifuge tube containing 0.5 cc. of 40 per cent trichloroacetic acid and a drop of 0.1N sodium thiosulphate solution. The grinder was rinsed twice with 0.5 cc. saline and the washings added to the centrifuge tube. The protein precipitate containing the organically bound I^{131} was separated from the supernatant containing the inorganic I^{131} by centrifugation. The protein was washed with 1.0 cc. of 5 per cent trichloroacetic acid, centrifuged again, and the liquid added to the first supernatant fraction. Plasma and the supernatant liquid were counted directly; the thyroid protein was dissolved in a strong potassium hydroxide solution and then counted.

In thyrotropin experiments 1, 2, and 3 of Table 2, only the iodine bound during the experiment was determined. Iodide was discharged from the thyroid by injecting 10 mg. potassium thiocyanate one hour prior to killing the animal and the entire gland counted.

The radioactivity measurements were made in the following manner. 1.0-cc. liquid samples were counted in standardized 10-cc., flat-bottomed vials. The base of the vial was placed in contact with a shielded and firmly fixed Geiger-Mueller tube through a hole in the lead shield cut exactly to size for the standard vial. The Geiger-Mueller tube was a brass-encased, gamma-ray counting tube of high efficiency (Sylvania, Type GG306). In this constant geometrical arrangement, one microcurie of I^{131} in 1.00 cc. produced 272 counts/second.

EXPERIMENTAL AND RESULTS

It was noted early in the course of the experiments that considerably more iodide was required to inhibit organic binding in the stock rats in our colony than had been required for the animals used by Wolff and Chaikoff. In experiment 1, Table 1, satisfactory inhibition was produced by 600 μ g. of iodide during a six and one-half hour experiment, and thiocyanate acted to remove the iodide block and increase the rate of synthesis of organic iodine compounds. In the subsequent experiments shown in Table 1, rats were used a number of days after a course of treatment with propylthiouracil, and while being maintained on a diet relatively low in iodine. Propylthiouracil was used to produce hyperplastic thyroid glands, and the low-iodine diet to maintain the hyperplastic state after the propylthiouracil was

TABLE 1. EFFECT OF THIOCYANATE ON THE ORGANIC BINDING OF IODINE IN RATS INJECTED WITH LARGE DOSES OF IODIDE

Expt. No.	Iodide injected μ g.	KSCN injected Mg.	Experi- mental period hours	Rat wt. gm.	Thyroid wt. mg.	Iodide conc. ratio thyr:se	Thyroid iodide mg./100 gm. thyroid tissue	Iodine organically bound during experiment μ g.
I	600	0	6.5	87	8.25	—	1.63	.152
	600	0		89	6.5	—	1.39	.06
	600	0		90	10.1	—	1.89	.0567
	600	7.5		89	8.15	—	0.291	.176
	600	7.5		86	8.0	—	0.348	.177
	600	7.5		87	9.2	—	0.522	.243
	600	7.5		87	9.1	—	0.345	.551
II	100	0	4.0	120	43.3	204.1	9.9	.097
	100	0		105	25.4	228.5	10.2	.056
	100	1.0		125	41.8	53.4	3.5	.15
	100	1.0		98	42.9	32.0	2.8	.13
	100	30.0		115	44.9	3.1	.45	.22
	100	30.0		145	33.3	3.0	.24	.14

Group II: Propylthiouracil 13 days, with low-iodine diet during last 36 hours. Continued on low-iodine diet and used 3 days after propylthiouracil stopped.

III	100	0	4.0	155	36.0	205.5	7.3	.17
	100	0		140	33.2	159.8	8.0	.099
	100	2.0		105	39.9	30.5	3.3	.069
	100	2.0		145	32.4	29.2	2.3	.22
	100	30.0		180	39.9	3.2	0.16	.35

Group III: Propylthiouracil 17 days, with low-iodine diet during last 2 days. Continued on low-iodine diet and used 3 days after propylthiouracil stopped.

IV	150	0	5.0	170	41.7	86.2	4.9	.08
	150	0		173	35.1	128.0	6.0	.155
	150	7.5		177	36.9	8.2	0.69	.419
	150	7.5		175	27.8	16.0	1.01	.33
	150	30.0		185	39.7	3.3	0.2	.19
	150	30.0		165	28.3	2.5	0.22	.17

Group IV: Propylthiouracil 23 days, with low-iodine diet during last 2 days. Continued on low-iodine diet and used 4 days after propylthiouracil stopped.

V	200	0	4.5	90	13.2	41.8	6.7	.051
	200	0		80	11.4	10.3	1.6	.061
	200	0		85	13.5	62.8	6.5	.064
	200	7.5		75	9.9	5.3	1.1	.083
	200	7.5		82	12.2	3.3	0.46	.32
	200	7.5		92	19.4	6.0	0.44	.23
	200	30.0		75	10.7	2.4	0.22	.066
	200	30.0		75	10.4	1.7	0.2	.061
	200	30.0		85	12.8	2.8	0.23	.102

Group V: Propylthiouracil 10 days, with low-iodine diet during last 4 days. Continued on low-iodine diet and used 12 days after propylthiouracil stopped.

stopped. The hyperplastic thyroid glands thus produced were readily inhibited by smaller doses of iodide than were required by non-hyperplastic glands. These enlarged glands were more like those studied by Wolff and Chaikoff, which were probably hyperplastic as a result of a low-iodine diet. The pretreated animals maintained thyroid:serum iodide ratios which were much higher than those of the untreated animals, and were in the range of the ratios reported by the previous workers.

Table 1 and Figure 1 demonstrate that, following a large dose of iodide, an inverse correlation existed between the iodide concentration in the thyroid and the quantity of iodine organically bound. Under the conditions of the experiment, organic binding was increased by thiocyanate.

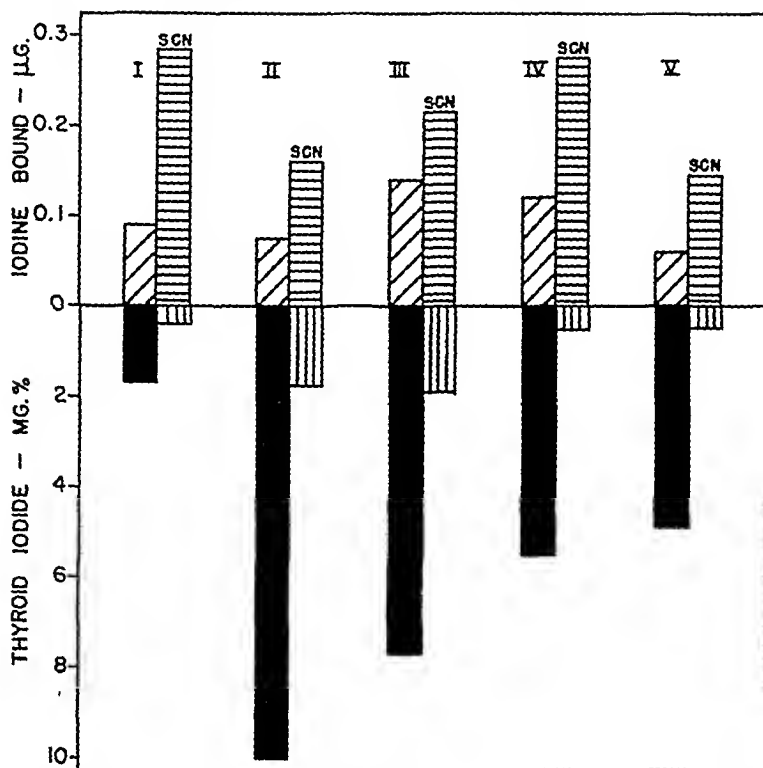


FIG. 1. Thiocyanate effect. Illustration of data from Table 1. The columns on the left for each experiment represent the animals which received no thiocyanate, and those on the right, the thiocyanate-treated animals. The thyroid iodide concentrations and the values for iodine organically bound have been averaged in each experiment for the thiocyanate group and the untreated group. The average bound iodine is represented by the columns above the line, and the average thyroid iodide concentrations by the columns below the line. It may be seen that a large thyroid iodide concentration is associated with a small amount of iodine bound, and that when the thyroid iodide is decreased with thiocyanate, iodine binding is increased.

In converse experiments, rats were injected with amounts of iodide which, though large, were not large enough to block iodine binding. A block was effected under these conditions, however, by pretreatment with thyrotropin. The data are shown in Table 2 and illustrated in Figure 2.

DISCUSSION

The experimental results indicate that high concentrations of iodide have a marked inhibiting effect on iodine binding by the thyroid, as reported by Wolff and Chaikoff in the rat, and as subsequently observed in man (Stanley, 1949). It further appears that the inhibition is a function of the iodide ion concentration in the thyroid gland. Smaller doses of iodide were required to produce inhibition in animals maintaining a higher thyroid:serum iodide ratio

TABLE 2. EFFECT OF THYROTROPIN ON THE ORGANIC BINDING OF IODINE IN RATS INJECTED WITH LARGE DOSES OF IODINE

Expt. No.	Iodide injected μ g.	Thyrotropin injected Mg.	Experimental period hours	Rat wt. gm.	Thyroid wt. mg.	Iodide conc. ratio thy:se.	Thyroid Iodide mg./100 gm. thyroid tissue	Iodine organically bound during experiment μ g.
I	42	0	6.5	33	—	—	—	.60
	42	0		33	—	—	—	.43
	42	15		32	—	—	—	.047
	42	15		35	—	—	—	.054
Group I: Thyrotropin injected 10 hours before iodide.								
II	45	0	6.5	50	—	—	—	.78
	45	0		65	—	—	—	.71
	45	15		70	—	—	—	.64
	45	15		50	—	—	—	.1
Group II: Thyrotropin injected 10 hours before iodide.								
III	35	0	6.25	38	—	—	—	.57
	35	0		32	—	—	—	.38
	35	0		33	—	—	—	.29
	35	15		32	—	—	—	.08
	35	15		38	—	—	—	.31
	35	15		30	—	—	—	.12
Group III: Thyrotropin injected 9½ hours before iodide.								
IV	42	0	6.5	31	4.5	—	0.22	.28
	42	0		33	4.5	—	0.51	.32
	42	0		32	5.0	—	0.43	.49
	42	15		30	5.0	—	0.54	.04
	42	15		32	4.9	—	0.83	.17
Group IV: Thyrotropin injected 10 hours before iodide.								
V	60	0	4.0	140	10.8	24.1	0.69	.553
	60	0		140	9.6	33.1	1.06	.52
	60	0		100	6.3	11.9	0.51	.22
	60	20		130	11.7	54.8	1.54	.108
	60	20		130	11.4	44.0	1.46	.051
	60	20		120	9.8	45.6	1.6	.083
Group V: 10 Mg. Thyrotropin injected 84 hours and 10 mg. 24 hours before iodide.								

than in those with a lower ratio. Suppression of the iodide concentrating mechanism by thiocyanate released the gland from the inhibiting effect, whereas increasing the iodide concentrating power of the gland with thyrotropin accentuated the iodide inhibiting action.

In the thiocyanate experiments, plasma iodide concentrations at the end of the experimental period were almost invariably above 35 μ g. per 100 cc., the concentration that inhibited binding in all the animals studied by Wolff and Chaikoff. There was essentially no difference in the plasma iodide values for the thiocyanate treated and non-treated groups. The increase in iodine binding in the thiocyanate treated animals, which can be correlated with the thyroid iodide concentration, cannot be correlated with the plasma iodide concentration.

It becomes of interest to inquire into the relationship between the iodide concentration in the thyroid and the quantity bound when the iodide values are below the inhibitory level. If results such as those shown in Figure 3 prove to be the usual relationship, it would appear that the rate of organic synthesis of iodine compounds increases with

increasing iodide concentrations. This would imply that a maximum value for iodine binding would be attained as thyroid iodide is increased, but that the further addition of iodide would then depress binding. An analysis of the data on the rat published by Wolff and Chaikoff (1948b) reveals that these are consistent with this concept, as are the data of Stanley on man.

The only action of thiocyanate on the thyroid gland that has been clearly demonstrated is a marked inhibition of the iodide ion concentrating power of the gland. When, as in Figure 4, organic synthesis

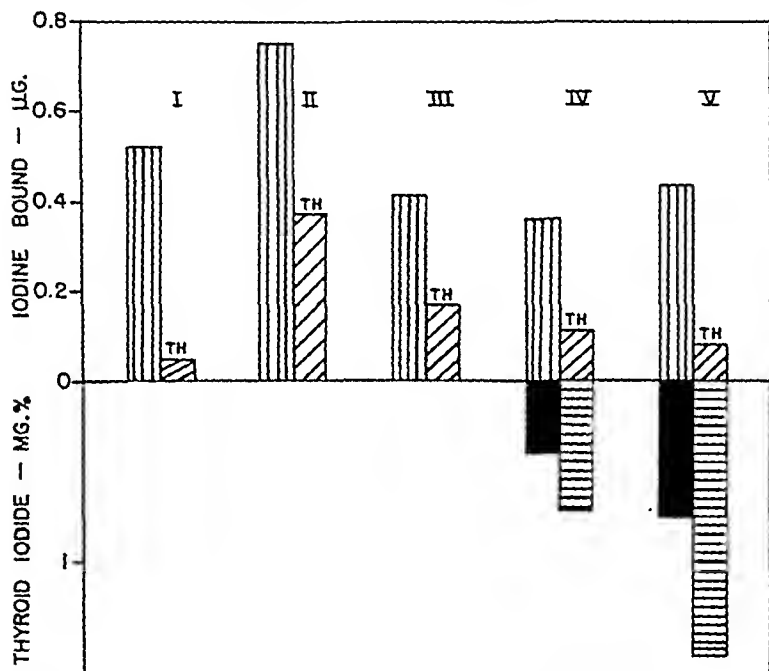


FIG. 2. Thyrotropin effect. Illustration of data from Table 2. Values have been averaged as for Figure 1, and columns arranged similarly. Thyroid iodide values were not determined in experiments I, II, and III.

is depressed by thiocyanate in the presence of low or moderately elevated plasma iodide values, it seems reasonable to relate the depression to the unavailability of iodide to the thyroid through the loss of its concentrating capacity.

It appears that even large amounts of thiocyanate cannot reduce the thyroid:serum iodide ratio below 1, and indeed, the ratio tends to remain somewhat above 1. When three rats weighing 100 ± 2 Gms. were each injected with 30 mg. of potassium thiocyanate, the thyroid:serum ratios one and one-half hours after the injection were found to be 1.4:1, 1.8:1, and 1.7:1. A high plasma level of iodide should therefore overcome the thiocyanate inhibiting effect on iodine binding if

this substance acted only on the iodide concentrating mechanism. The prevention of thiocyanate goiter with iodide (Astwood, 1943) suggests that such is the case. Experimental results at times, however, suggested the possibility that large doses of thiocyanate could in addition affect organic synthesis directly. Such suggestion appears both in some of the data in Table 1, and in the data shown in Figure 5.

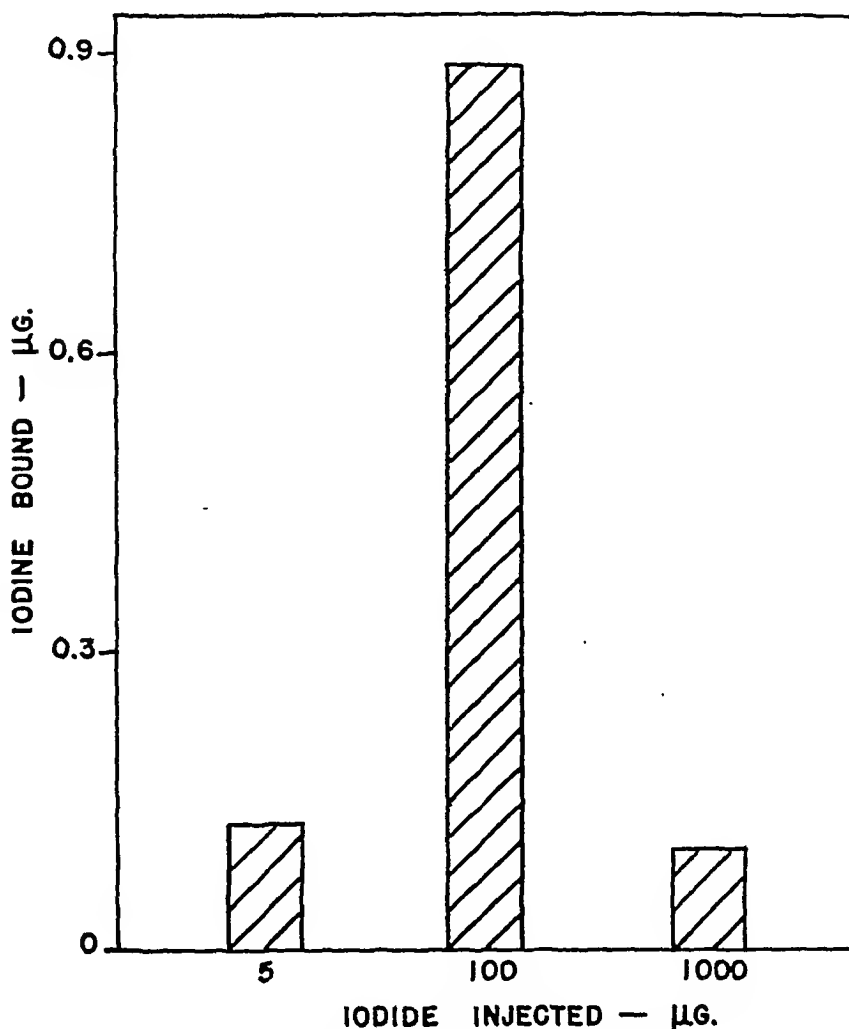


FIG. 3. The influence of iodide dosage on the amount of iodine bound. Rats weighing 82 ± 5 Gms. were injected with 5.0, 100, or 1000 µg. of iodide labelled with I^{131} . After $6\frac{1}{2}$ hours, the animals were injected with potassium thiocyanate to discharge thyroid iodide, killed 45 minutes later, and the radioactivity in the glands measured. The column for the 1000 µg. animals is probably about 33 per cent too high due to iodide remaining in the thyroid, as estimated from the results of iodine fractionations in other animals under the same conditions. Each column represents the average of two animals.

Figure 5 illustrates the effect of various amounts of thiocyanate on iodine binding in the presence of large amounts of iodide. When 100 µg. of iodide was used, an amount which was insufficient to block binding, a progressive decrease in binding occurred with increasing

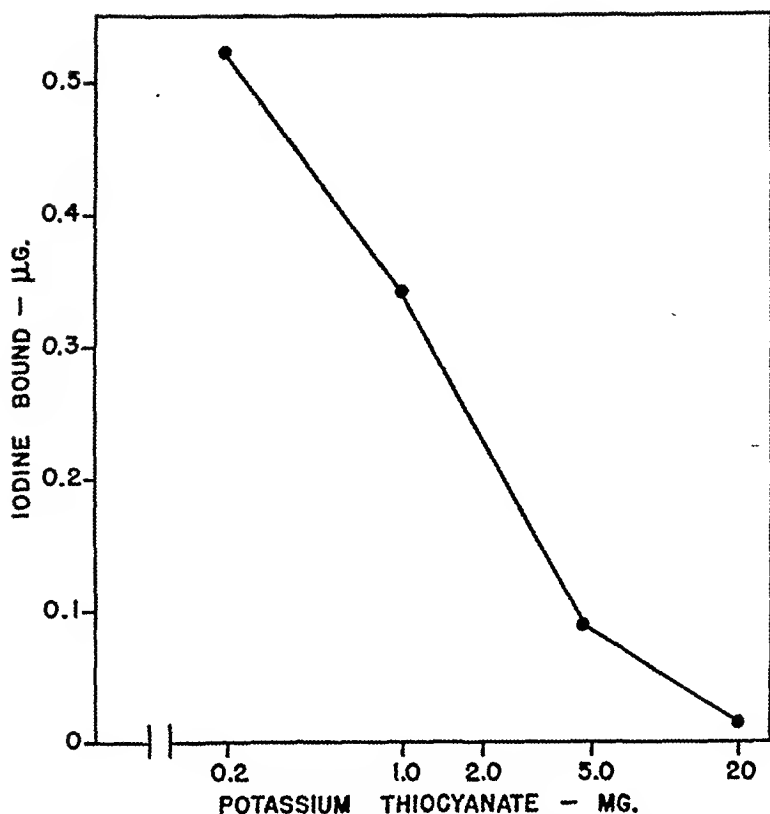


FIG. 4. Relationship of thiocyanate dosage to the quantity of iodine bound. Rats weighing 86 ± 6 Gms. were each injected with 15μ g. of labelled iodide and with the doses of potassium thiocyanate indicated on the abscissa. The same procedure was followed as in Figure 3. Each point represents the average of two animals.

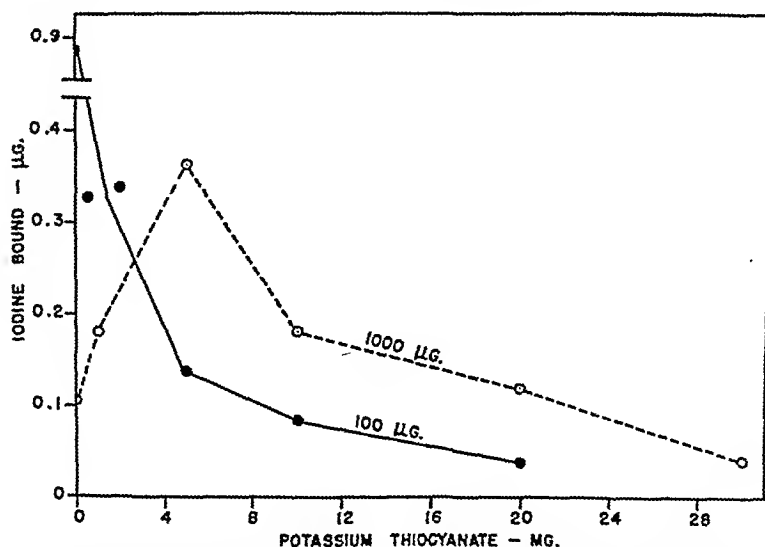


FIG. 5. Relationship of thiocyanate dosage to the quantity of iodine bound in the presence of large amounts of iodide. The rats weighed 85 ± 12 Gms. and the same procedure was followed as for Figure 3. Each point is the average of two animals.

amounts of thiocyanate. The results obtained with 1000 μ g. of iodide indicate that this dose blocked binding and that the addition of thiocyanate acted to release the block, as in the experiments described earlier. However, with the larger doses of thiocyanate (20 and 30 mg.), binding was again depressed. Extremely low values were obtained for bound iodine in the group receiving 100 μ g. of iodide and 20 mg. of potassium thiocyanate, and in the group treated with 1000 μ g. of iodide and 30 mg. of potassium thiocyanate. It is difficult to account for such low values on the basis of thyroid iodide concentrations since these would be adequate for more rapid binding even if one assumed a thyroid: serum ratio as low as 1.

SUMMARY

The inhibition of iodine binding by the thyroid which occurs in the rat following a large dose of iodide is shown to be directly related to the iodide concentration in the thyroid gland. The usual effects of thiocyanate and of thyrotropin on the rate of organic binding of iodine by the thyroid can be reversed by the addition of large amounts of iodide. It is believed that the influence of these substances on the iodide concentrating power of the gland explains the paradoxical effects.

ACKNOWLEDGMENTS

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STUDIES ON THE FAT-MOBILIZING FACTOR OF THE ANTERIOR PITUITARY GLAND¹

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ON THE bases of chemical and physiological evidence there has been general acceptance of six well-defined anterior pituitary hormones: growth hormone, thyrotrophin, corticotrophin, luteotrophin (lactogenic hormone), follicle-stimulating hormone, and luteinizing hormone. Anterior pituitary preparations have, however, been noted to cause a number of metabolic effects variously described as diabetogenic, glycotrophic, glycostatic, fat mobilizing, respiratory quotient lowering, ketogenic, pancreatotrophic, etc. It would be desirable to define more closely the hormones responsible for these various effects so as better to characterize the secretions of this complex endocrine gland.

This study deals with the hypophyseal factor which leads to an increase in liver fat; it was undertaken with the aim of determining whether this principle could be identified with any one of the six well-characterized hormones, or whether evidence could be adduced indicating a separate factor.

METHODS

The fat-mobilizing activity of a number of pituitary preparations was studied by the method of Campbell (1938). Immature female mice of the Swiss strain weighing 18 to 22 grams, fed on Purina mink chow, were given a single subcutaneous injection of the test substance and then fasted until they were killed with chloroform seven hours later. After injection the animals were placed in cages the floors of which were covered with wood shavings and permitted access to water. The room temperature averaged 25°C.

Immediately after death a 500-mg. portion of the liver was ground with approximately 4 gm. of anhydrous sodium sulfate in a dry mortar as described by Weil and Stetten (1947). The powdered preparation was then put into a 50-cc., clean, dry, screw-cap vial, the cap of which was lined with tinfoil and seated in cork. Thirty cubic centimeters of chloroform was added and the screw cap sealed. The preparation was then either turned end over end by a mechanical device at 50 to 60 revolutions per minute for sixteen hours or allowed to stand for seventy-two hours with intermittent shaking by hand.

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Ten cubic centimeters of the supernatant fluid was then filtered into a tared beaker, evaporated to dryness in a hot-air oven and weighed.

This method showed good correlation with the Soxhlet intermittent type of fat extraction on duplicate samples of mouse and rat livers of various fat concentrations. It had the advantage of simplicity of equipment and ease of operation.

Every effort was made to keep the preparation free from water as the presence of water prevented efficient extraction. Excess water could be determined by placing a pinch of anhydrous copper sulfate into the solution. A change of color to blue indicated water contamination.

Adrenalectomy was performed under ether anesthesia through a lumbar approach. All animals were discarded if the capsule of the adrenal was broken during removal. Adrenalectomized animals were given 0.9 per cent NaCl as drinking water and were used one week postoperatively. Thyroidectomy was done with ether anesthesia under a dissecting microscope. Propylthiouracil was given in a concentration of .03 per cent admixed with the food for two weeks prior to assay; this led to marked thyroid enlargement indicating an appreciable if not complete inhibition of thyroid hormone formation.

The soluble anterior pituitary extracts were prepared for injection by solution in physiological saline; the crude powders were suspended in saline with a homogenizer. All injections were given subcutaneously. When two separate substances were administered, they were given in different sites.

Each anterior pituitary preparation was tested over a wide dosage range; the resulting liver fat concentration was then plotted against the log of the dose. With most preparations a roughly linear dose-response relationship was noted over a limited range, and the potencies of different preparations were compared by reference to this portion of the curves.

RESULTS

About 500 separate determinations of liver fat concentration were done by the method described. The liver fat concentration of sixty normal control fasted mice ranged from 4 to 6 per cent and averaged 4.96 per cent. No difference was noted in the control animals whether untreated or injected with 1 cc. of 0.9 per cent NaCl solution.

All anterior pituitary preparations tested showed some activity, though the potencies varied between wide limits. The quantities of fat-mobilizing factor in the various preparations tested are shown in Figures 1 to 4.

Figure 1 represents the activity of acetone-dried pituitary powders of various species. These were similar in activity with the possible exception of pork pituitary, which was somewhat more active. Figures 2, 3, and 4 illustrate the fat-mobilizing effect of preparations of the pituitary which had been further purified. These refined preparations have been catalogued according to their activity into high, medium, and low potency groups.

A refined pituitary substance, Antuitrin T-607, was the most potent of the preparations tested. It was noted with this preparation

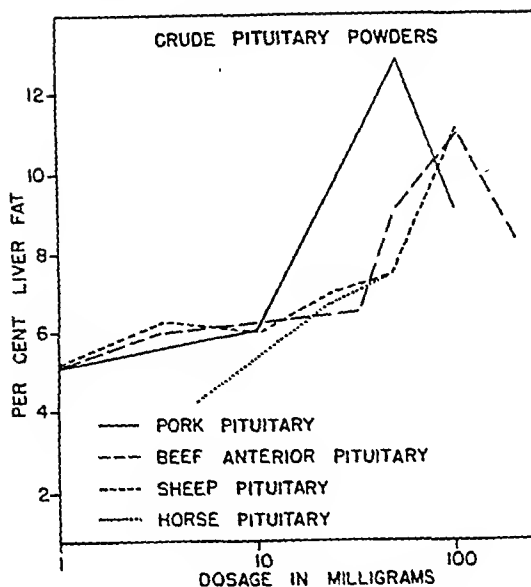


FIG. 1. Fat-mobilizing activity of crude, defatted pituitary powders. All preparations except beef anterior pituitary were derived from the whole pituitary.

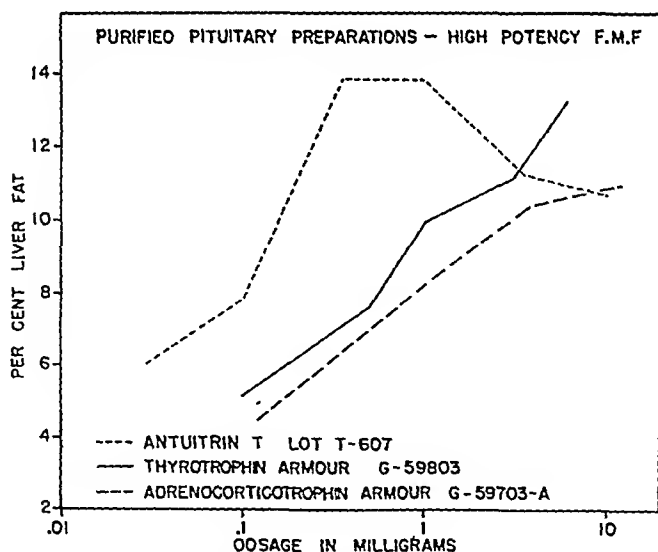


FIG. 2. Antuitrin T-607 contained the highest content of fat-mobilizing factor of the preparations tested. Thyrotrophin G-59803 possessed a high content of thyrotrophin and adrenocorticotrophin with a low assay of other factors. Adrenocorticotrophin G-59703-A contained only slightly higher adrenocorticotrophic activity than the above thyrotrophin. The latter two preparations had been subjected to sterilization filtration but still contained slightly greater potency of fat-mobilizing factor than the preparations shown in Figure 3.

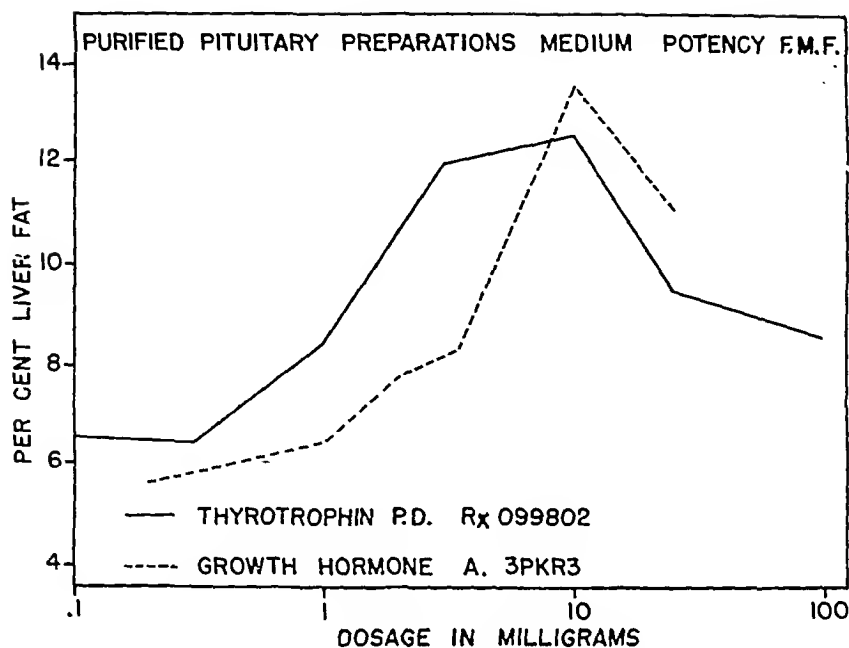


FIG. 3. Thyrotrophin (Parke-Davis R_x 099802) possessed the highest thyrotrophic activity of any preparation tested.

Growth Hormone 3PKR3. Purified growth hormone (Armour).

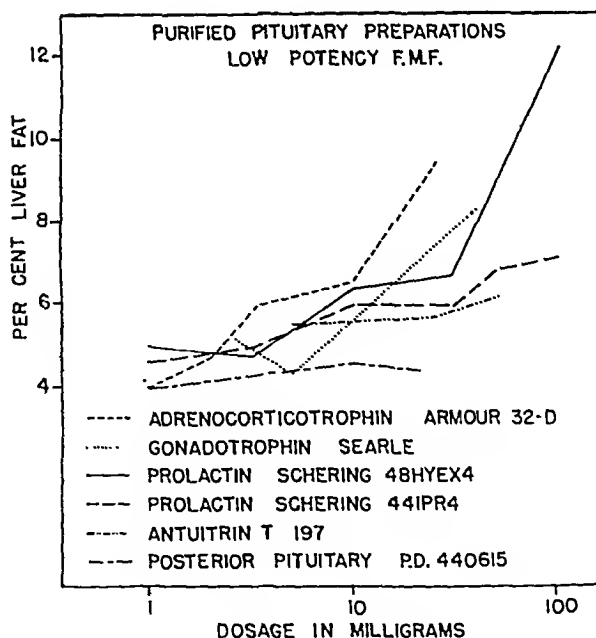


FIG. 4. Adrenocorticotrophin 32-D contained the highest adrenocorticotrophic activity of any preparation tested . . . (78 per cent of Armour Standard) with a moderately high assay of Prolactin ($2\frac{1}{2}$ I.U./Mgm.). Schering's Prolactin 48HYEX4 contained 20 I.U./Mgm. and Prolactin 441PR4 contained 7.5 I.U./Mgm.

that about 30 per cent of the activity was lost in five days when solutions of the material were kept at room temperature. *B. subtilis* was cultured from these solutions and may have accounted for the change of activity. The tests on the other preparations were made on freshly prepared solutions.

Saline suspensions of posterior pituitary showed no activity in the dosages used. Pitressin, 1 cc., (Parke-Davis), was quite toxic to the

TABLE 1. ADRENAL EFFECT ON FAT-MOBILIZING FACTOR

Number of animals	Per cent liver fat
<i>Intact</i>	
3 Lipo-Adrenal Cortex, 1 cc.....	5.53
1 Lipo-Adrenal Cortex, .3 cc.....	6.48
1 Lipo-Adrenal Cortex, .1 cc.....	4.88
2 Eschatin, 1 cc.....	6.09
2 Sesame Oil, .25 cc.....	4.68
<i>Adrenalectomized—7 Days Post-Operative</i>	
3 Uninjected.....	4.36
2 Antuitrin T*, 2.5 mg.....	5.61
2 Antuitrin T*, .5 mg.....	6.15
1 Thyrotrophin (Parke-Davis), 20 mg.....	3.00
2 Lipo-Adrenal Cortex, .5 cc.....	3.48
2 Desoxycorticosterone Acetate in Sesame Oil, 2.5 mg.....	5.2
3 Lipo-Adrenal Cortex, .5 cc.+Antuitrin T*, 2 mg.....	9.42
2 Lipo-Adrenal Cortex, .25 cc.+Antuitrin T*, 2 mg.....	8.73
4 Lipo-Adrenal Cortex, .05 cc.+Antuitrin T*, 2 mg.....	7.18
2 Lipo-Adrenal Cortex, .01 cc.+Antuitrin T*, 2 mg.....	8.37
2 Eschatin, 1 cc.+Antuitrin T*, 2 mg.....	8.79
2 Eschatin, .5 cc.+Antuitrin T*, 2 mg.....	5.94
8 Desoxycorticosterone Acetate in Sesame Oil, 2.5 mg.+Antuitrin T*, 2 mg.....	7.83
2 Sesame Oil, .25 cc.+Thyrotrophin (Parke-Davis), 20 mg.....	5.12

* Antuitrin T, Lot T-607 (Parke-Davis & Company).

mice and caused a slight rise in liver fat (6.24–6.6 per cent liver fat). These values are not considered significant because of the possible effect on the liver of the preservative, chloretoone.

The maximum response was higher with some preparations than with others, suggesting that there might have been other factors modifying the fat-mobilizing effect. When the dosage was increased above that which gave a maximal increase in liver fat, the response progressively declined. This phenomenon is unexplained.

Under the conditions of this experiment adrenalectomy inhibited completely the response to the fat-mobilizing factor (Table I). The response of the adrenalectomized animals to this factor was restored by adrenal cortical extract. Lipo-Adrenal Cortex (Upjohn) was in excess of fifty times as effective as the aqueous adrenal extract used (Eschatin—Parke-Davis) in this particular function. The mobiliza-

tion of the oil in the preparation was found not to be the responsible agent as the injection of sesame oil alone was without effect. However, without added fat-mobilizing factor the adrenal cortical hormones did not produce fatty liver in either the intact or the adrenalectomized animal. The injection of Lipo-Adrenal Cortex with fat-mobilizing factor in the intact animal produced no substantial change in the shape of the response curve, except perhaps for a slight depression of the effect in the higher dosage range (Fig. 5). When desoxycorticosterone

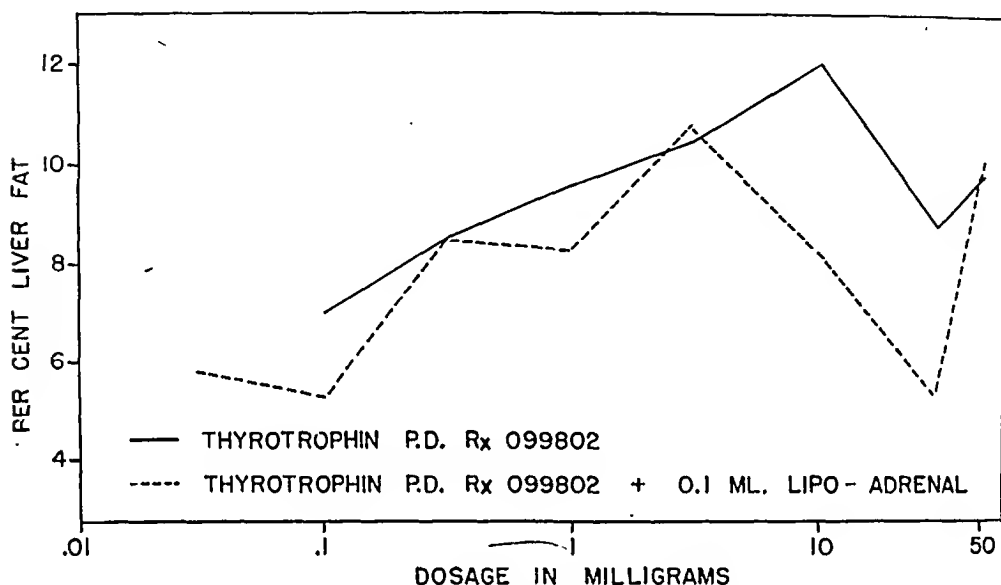


FIG. 5. The effect of adrenal cortical substance on fat-mobilizing factor activity in the intact fasted mouse. There was generally a slight depression of effect noted particularly in the higher dosage range.

acetate was used as replacement therapy in the adrenalectomized animals, a variable response to fat-mobilizing factor was obtained. Desoxycorticosterone acetate produced no change in the liver fat of adrenalectomized or intact animals.

The interrelationships of the thyroid were studied in the same manner. Thyroxine in maximal doses of 1 milligram did not induce fatty livers in intact fasted animals. Neither treatment with propylthiouracil nor surgical thyroidectomy caused an increase in liver fat by themselves; these procedures did not abolish the liver fat response to injected pituitary extracts.

No increase in liver fat was obtained with the following materials: desoxycorticosterone acetate, 5 mg. in sesame oil; stilbestrol, 5 mg. in sesame oil; epinephrine hydrochloride, .4 mg. (aqueous); regular insulin, .5 u.; testosterone acetate, 25 mg. in sesame oil, or; crystalline progesterone, 10 mg. in sesame oil.

Doses of choline chloride up to 2.5 mg. were shown to cause no

depression of fatty liver response to fat-mobilizing factor in accord with the observation of MacKay and Barnes (1938).

The effect of cold was studied by exposing the animals to an environmental temperature of 6°C. for seven hours. This caused a moderate increase in liver fat (averaging 7.34 per cent), as described by Leblond, Van Thoi, and Segal (1939). When fat-mobilizing factor was given during the period of cold exposure, the liver fat showed a normal response. When wire-bottom cages were used in the experiments which were conducted at room temperature, a variation in response was observed. This might also indicate some influence of exposure upon the effect.

DISCUSSION

Earlier investigators found the fat-mobilizing factor to be absent in the anterior pituitary (Coope and Chamberlain, 1925). These negative results might have been due to the use of preparations such as Antuitrin T-197, which has a very low potency of this factor. Best and Campbell (1936) showed fat-mobilizing effect to be present in the anterior pituitary.

Depression of fat mobilization in the rat by adrenalectomy, as described by Fry (1937), MacKay (1937) and others, seems to hold also in the mouse. The restoration by adrenal cortical hormone (and yeast) of the ability of phosphorus to cause fatty livers in adrenalectomized rats has been described by Verzar and Laszt (1936).

The lack of correlation of the fat-mobilizing potency with the adrenocorticotrophic activity of the preparations used, and the failure of large doses of cortical extracts to produce fatty livers in the intact mouse, indicate that the fat-mobilizing factor is not identical with adrenocorticotrophin, nor, by similar reasoning, with thyrotrophin. Likewise, the inability to correlate the fat-mobilizing factor with the potency of any of the other better known trophic hormones indicates that it is distinct from them. However, since adrenal cortical secretion is necessary for the immediate mobilization of fat, the adrenal must, in some way, facilitate the mobilization of depot fat. These findings might be associated in a general way with the known interplay of glycogen on body fat deposition (Wertheimer and Shapiro, 1948) and the glycogenic effect of adrenal cortical hormone. The possibility thus presents itself that the adrenal by its glycogenic function acts on immediate fat mobilization by priming the body fat depots for the action of fat-mobilizing factor, analogous to the preparation of the breasts by ovarian function for institution of lactation by pituitary extract (Stricker and Greuter, 1929).

Fat mobilization in response to stress as suggested by Leblond (1939), who considered it as a part of the adaptation syndrome of Selye, was not observed in these experiments. Substances which are known to cause profound adrenal stimulation, i.e. stilbestrol, insulin,

and epinephrine, were without effect in producing fatty livers, nor did adrenal cortical hormone alone cause the effect. Progesterone, testosterone, and stilbestrol were found not to be effectors in the mechanism of fat mobilization.

Recently Szego and White (1948) studied the effect of growth hormone (Wilhelmi, Fishman, and Russell, 1948) on liver fat. They found that adrenalectomy did not inhibit fat mobilization in 48-hour fasted male mice. This might indicate that with prolonged fasting another mechanism of fat mobilization, independent of the adrenals, could be operative. The absence of depot fat in adrenalectomized animals, as mentioned by Wertheimer (1948), would support the view that there is some utilization of body fat in such animals.

The lack of thyroid effect on immediate fat mobilization would support the view of Himsworth (1947) that the effect of the thyroid in production of fatty liver is nutritional in origin.

The inability of adrenal cortical extract to cause increased fat mobilization by fat-mobilizing factor in the intact animal indicates that the effect of the adrenals on fat mobilization is adequate for maximal stimulation in the animal with normally functioning adrenals. The slight depression of liver fat observed in these animals might be due to increased metabolism of fats in the livers of such animals with subsequent rapid utilization.

SUMMARY

The effect of various anterior pituitary preparations on the liver fat concentration of fasted mice has been investigated using a simplified method for the determination of total fat in the liver. The deposition of fat in the liver appeared to be caused by a separate substance, termed the fat-mobilizing factor which could not be identified with any one of the well-recognized anterior pituitary hormones. Though the adrenals are essential to the action of this factor, the effect is not due to adrenocorticotrophin nor is the effect mediated by the adrenal cortex.

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The author greatly appreciates the helpful guidance of Dr. E. B. Astwood in this problem.

We are indebted to Dr. David Klein of the Wilson Laboratories for the acetone-dried pituitary powders, to Dr. D. A. McGinty of Parke-Davis and Company for samples of Antuitrin T, and to Dr. John R. Mote of the Armour Laboratories for preparations of corticotrophin, thyrotrophin, and growth hormone. The Lipo-Adrenal Cortex was kindly supplied by Dr. H. F. Hailman of the Upjohn Company.

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ASCORBIC ACID IN THE PITUITARY OF THE RAT¹

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THE RECENT work of Long (1947a, b) and Sayers and Sayers (1948) has demonstrated that exposure to stress or injection of ACTH results in the depletion of approximately 40–50% of the ascorbic acid normally present in the adrenal cortex. While the specific role of this substance in the formation and release of the adrenal corticoids is not yet known, it has been generally accepted that the loss of this vitamin is associated with the release of the cortical steroids. Thus it has been postulated that the changes in the concentration of ascorbic acid in the adrenal cortex may be used as an index of adrenal cortical activity (Long, 1947b).

It has also been reported that the hypophysis contains a high concentration of ascorbic acid (Giroud, 1938; King, 1939) and that a decrease in ascorbic acid content occurs in the pituitary of the female rabbit following copulation (Pincus, 1947). These reports led us to examine the vitamin C concentration of the pituitary gland of the rat and rabbit after subjecting the animals to various stimuli. It was felt that if changes were found in the vitamin C concentration of the pituitary, these alterations could be used as an index of activity in the hypophysis.

Secretion of the pituitary hormone was modified by several different procedures. Rabbits were mated or stimulated cervically with a faradic current of five volts to effect the release of gonadotropins and killed one hour later. Rats were subjected to (a) thiouracil² treatment in order to increase the thyrotropic activity of the pituitary gland, (b) electrical stimulation of the cervix during estrus to produce release of gonadotropins and (c) electrical stimulation of the vagus to release posterior pituitary hormone. The rate of secretion of ACTH was increased by (a) adrenalectomy, (b) exposure to cold ($3 \pm 1^\circ\text{C}$.) for one and twelve hours, and (c) intraperitoneal injection of epinephrine and histamine diphosphate. In the latter instance, the animals were killed 30 minutes after injection. Inhibition of ACTH secretion

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² Thiouracil was obtained through the courtesy of Dr. Stanton Hardy, American Cyanamide Company, Lederle Laboratories Division, Pearl River, New York.

was attempted by injecting cortin³ and ACTH.⁴ Normal animals of appropriate age and weight were used as controls.

All animals were killed by a sharp blow on the head. The pituitary gland was removed in less than 30 seconds, weighed and triturated in 6% trichloroacetic acid. Ascorbic acid content was determined according to the method of Roe and Keuther (1943).

Examination of the data (Table 1) shows that the amount of

TABLE 1. CONCENTRATION OF ASCORBIC ACID IN THE PITUITARY OF THE NORMAL AND TREATED RAT

No. of rats	Sex	Body wt. grams	Treatment	Pituitary weight mgm.	Ascorbic acid mgm. %
19	♀	81	—	3.9	112
15	♀	171	—	8.9	131
21	♀	216	—	12.2	145
7	♀	223	Cold—1 hour	13.3	145
4	♀	210	Cold—12 hours	13.5	140
16	♀	173	Adren. 24 hours	9.5	132
7	♀	184	Adren. 72 hours	9.8	135
8	♀	201	Histamine		
			10 mgm./100 gr. body wt.	11.5	143
9	♀	212	Epinephrine		
			0.2 mgm./100 gr. body wt.	11.8	140
4	♀	236	Cortin		
			10 dog units daily/6 days	14.9	157
5	♀	223	Cortin		
			10 dog units daily/11 days	13.4	145
3	♀	197	ACTH		
			2 mgm. daily/6 days	9.5	148
10	♀	196	Thiouracil		
			0.1% for 12 weeks	12.1	156
6	♀	220	Electrical stimulation of the cervix	13.4	143
16	♀	205	Electrical stimulation of the vagus	10.4	141
16	♂	218	—	7.4	139
17	♂	226	Electrical stimulation of the vagus	7.0	128

vitamin C in the hypophysis increases with age. It was found that the pituitary gland of the young female rat weighing 81 grams contained 112 mgm.% of ascorbic acid while in older rats weighing 171 and 216 grams, the concentration increased to 131 mgm.% and 145 mgm.% respectively. However, under the experimental conditions designed to alter the rate of secretion of ACTH, TSH, gonadotropins and posterior pituitary factor, no significant changes were found in the concentration of ascorbic acid in the pituitary gland.

Ascorbic acid determinations were also carried out on the pituitaries of female rabbits. Three rabbits killed one hour after mating showed a concentration of 113 mgm.% vitamin C in the pituitary gland while an average of 114 mgm.% was found in the pituitary gland of 5 control animals. In addition, no significant change was

³ Cortin was obtained through the courtesy of Parke-Davis Company.

⁴ ACTH was obtained through the courtesy of Armour & Company, Chicago, Illinois.

noted in ascorbic acid level of the hypophysis of three rabbits after electrical stimulation of the uterine cervix.

SUMMARY AND CONCLUSIONS

The normal concentration of ascorbic acid in the pituitary glands of adult rats remains constant. Experiments designed to produce changes in the content or production of TSH, ACTH, gonadotropins and posterior pituitary hormone failed to change significantly the concentration of ascorbic acid. No changes from normal were noted in the ascorbic acid concentration of the pituitaries of rabbits following mating or electrical stimulation of the uterine cervix.

An increase in ascorbic acid concentration of the pituitary gland was observed with increase in age of the rat.

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CYTOTOXIC ACTION OF HORMONES OF THE ADRENAL CORTEX ACCORDING TO THE METHOD OF UNSTAINED CELL COUNTS

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THE REACTIONS of cells to x-rays (Schrek, 1946) and nitrogen mustard (Schrek, 1947) have been studied previously by the method of unstained cell counts. This method has been used in this work to determine the effect of extracts and hormones of the adrenal cortex on cells of the thymus and of the bone marrow.

METHODS

The thymic gland or the bone marrow from the femurs of a rabbit was removed and placed in a small amount of Ringer's solution containing 5% isotonic phosphate buffer pH 7.6. The organs were chopped up with a fine pair of scissors and were then passed through a Seitz filter equipped with an 80 mesh Monel metal wire gauze. The cellular suspension thus obtained was washed once and resuspended in a mixture of equal parts of phosphate-Ringer's solution and rabbit serum.

The suspension was distributed into small test tubes in 0.4 ml. amounts. The reagent in various dilutions was added in 0.1 ml. amounts. The test tubes with the mixtures were placed in a water bath at 37°C. and shaken at the rate of 150 times per minute.

The reagents consisted of commercial extracts of the adrenal cortex and also preparations contributed by several laboratories. The commercial preparations included Lipo-Adrenal Cortex of the Upjohn Company and Eschatin of Parke, Davis and Company. Dr. W. J. Haines of the Upjohn Company generously contributed a dry extract of the adrenal cortex and the pure adrenal steroids, corticosterone and 17-hydroxycorticosterone. Adrenocorticotrophic hormone of the pituitary gland was provided through the courtesy of Dr. C. H. Li and Dr. John C. Mote.

Dilutions of Lipo-Adrenal Cortex and of desoxycorticosterone were made with peanut oil, sesame oil or serum. Eschatin and the adrenocorticotropin were diluted with Ringer's solution.

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The pure adrenal cortical steroids and the extract of the adrenal cortex was dissolved in 95% ethyl alcohol, and diluted with distilled water so that the final concentration of alcohol was 1%. In some experiments the hormones or extracts were diluted with 95% alcohol, the dilutions were then distributed to test tubes and the alcohol evaporated in vacuo at room temperature.

To count the number of viable cells, 3.5 ml. of a solution of safranin 1:4000 in Tyrode's solution was added to the 0.5 ml. of suspension in a test tube. A drop of the mixture was placed in a hemocytometer and the unstained, stained and red blood cells were counted. The number of unstained cells per micromilliliter was then calculated.

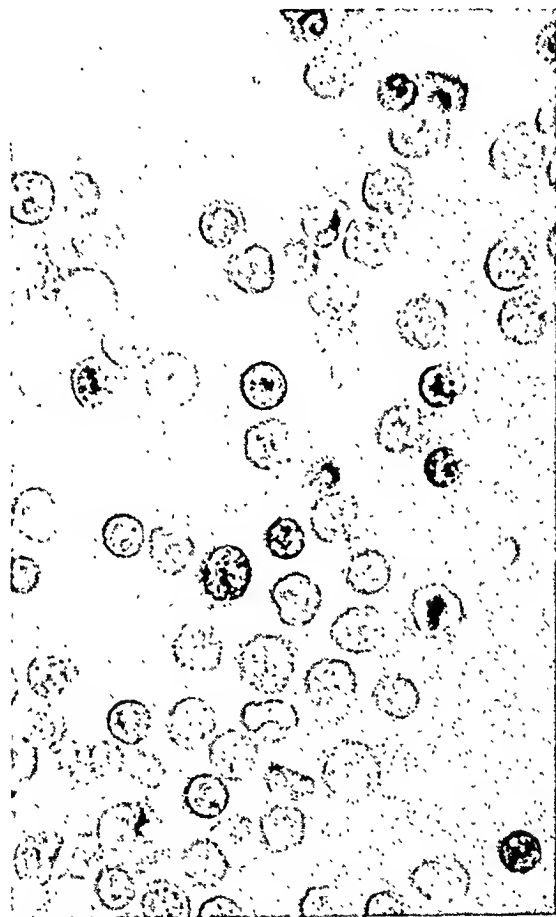


FIG. 1. Cells in a fresh suspension of a rabbit thymus. The suspension has been mixed with a safranin solution. Most of the cells are unstained, i.e. viable. A few cells are stained, i.e. dead. There is also an occasional erythrocyte. ($\times 1000$).

Effect of corticosterone on cells of the thymus. In a typical experiment the cells of a rabbit thymus were suspended in a medium consisting of homologous serum and phosphate-Ringer's solution. To a small quantity of the cellular suspension was added safranin, and a drop of the mixture was placed in a hemocytometer.

About 82% of the cells were (as in fig. 1) colorless, translucent

and round with fine granules throughout the cell. These cells which resisted staining with safranine may be considered as viable. The reasons for this assumption are discussed in a previous paper (Schrek, 1943).

About 14 per cent of the cells in the fresh suspension were stained red with the basophilic safranine as can be seen in fig. 1. The nuclei of these cells were deeply stained and had a thin nuclear membrane and fine chromatin granules. The ability of these cells to stain rapidly and intensely indicated that these cells were dead. They were pre-



FIG. 2. Cells of rabbit thymus in a suspension incubated 24 hours at 37°C and then mixed with a safranine solution. About half of the cells are unstained. The stained cells have either pyknotic or leptochromatic nuclei. ($\times 880$).

sumably killed in the preparation of the suspension. It is to be noted that only under certain exceptional experimental conditions was there any difficulty in differentiating between the completely colorless unstained cells and the stained cells.

In addition there were a few red blood cells which were unstained, but which were characterized by their yellowish color, their sharp contour and their lack of granularity.

In this particular experiment, the suspension had 231.5 unstained, 38 stained and 11 red blood cells per micromilliliter (table 1).

The suspension was distributed to test tubes in 0.4 ml amounts and varying dilutions of corticosterone in 1% alcohol was added in

0.1 ml amounts. The mixtures were incubated at 37°C. At periodic intervals viable cell counts were made by adding safranin (3.5 ml) to the suspension in a test tube and counting the unstained cells in a hemocytometer.

Immediately after the addition of hormone in 3- to 30-microgram amounts no appreciable change in the viable cell counts could be observed. The steroid did not have any immediate perceptible effect on the number of viable cells in the suspension.

In the control suspension incubated for 21 hours at 37°C, the number of viable cells decreased from 231.5 to 101.5 which is 43.8% of the

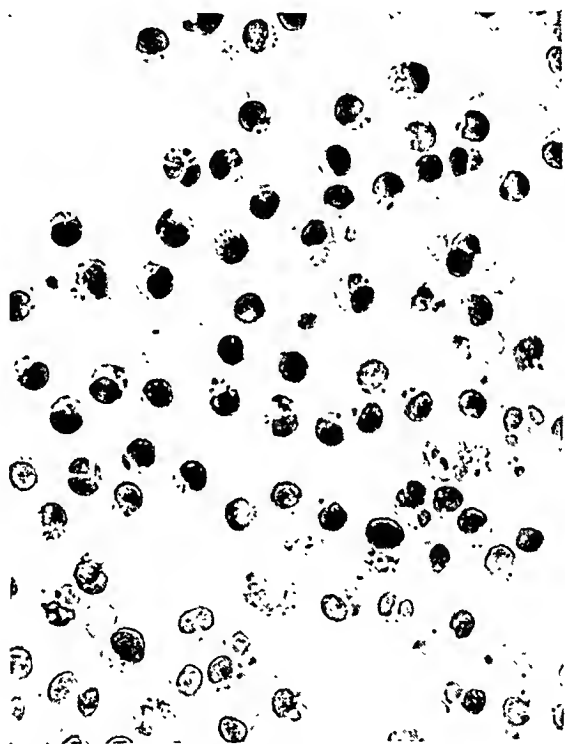


FIG. 3. Cells of a rabbit thymus in a suspension irradiated with 1000 r and incubated 24 hours at 37°C. Nearly all the cells have pyknotic nuclei. The same type of cells is obtained in suspensions treated with corticosterone. ($\times 880$).

original. In the suspension containing ethyl alcohol (0.2% final concentration), the same number of cells (101.0) survived. Evidently, the solvent used was not toxic to the cells.

Incubation of the suspension with 0.03 microgram of corticosterone had a small but definite toxic effect with the reduction of the viable cells to 71.5 as compared to 101.0 for the control. It appears that a perceptible effect was produced by 0.03 microgram in the presence of 116 million viable cells. Previous *in vitro* experiments have shown few reagents which are biologically effective in such minute quantities.

With a dosage of 0.3 microgram, fewer cells (only 61.3 or 26.5% of the original) survived the 21 hours of incubation. With 3 micrograms, the toxicity was increased further. But even this high dosage, 100 times the minimal, permitted 37.2 or 16.1% of the cells to survive the incubation. It seems that the hormone is only feebly toxic, in spite of the fact that it produces an effect even in low concentrations.

Additional observations at 27 and at 45 hours confirmed the findings obtained at 21 hours. Other experiments were conducted without the presence of alcohol and the results were substantially the same.

The table also shows the variations in the counts of viable cells. The average deviations for all counts in the table was 10% with vari-

TABLE 1. EFFECT OF CORTICOSTERONE ON SUSPENSION OF CELLS OF THE RABBIT THYMUS

Procedure: Test tubes with 0.2 ml. cellular suspension in phosphate Ringer's solution, 0.2 ml. rabbit serum, and 0.1 ml. corticosterone in 1% ethyl alcohol were incubated at 37°C. Counts were made after diluting the suspension with 3.5 ml. safranin in Tyrode's solution.

Hours of incubation	Final concentration		No. of viable cells per μ ml.				
	Alcohol %	Steroid μ g.	Average count	Average deviation %	% of control		Actual counts
					Based on original count**	Based on daily control***	
0	0	0	231.5*	6.6	100		262-215-225-224
21	0	0	101.5	9.1	43.8		103-83-105-115
	0.2	0	101.0	6.4	43.6	100.0	106-100-89-109
	0.2	3	37.2	2.0	16.1	36.9	36.5-38
	0.2	0.3	61.3	2.0	26.5	60.8	62.5-60
	0.2	0.03	71.5	2.1	30.9	70.9	73-70
	0.2	0.01	101.0	17.8	43.6	100.0	83-119
45	0	0	26.5	18.9	11.4		23-36-27-20
	0.2	0	46.0	25.0	19.9	100.0	55.5-59.5-35.5-33.5
	0.2	3	5.4	3.7	2.3	11.6	4.8-5.6
	0.2	0.3	6.5	1.5	2.8	14.1	6.6-6.4
	0.2	0.03	29.3	16.0	12.7	63.8	24.5-34
	0.2	0.01	32.3	8.4	14.0	70.4	29.5-35

* The counts show the number of cells in the original suspension. The final concentration of viable cells in the 0.5 ml. mixture was 92.6 cells per μ ml. or 92,600 per cu. mm. The total number of viable cells in the mixture was 116×10^4 .

** This column shows the percentages of viable cells surviving after incubation and treatment with the various reagents. These percentages are used as ordinates in graphs similar to fig. 4.

*** The differences between these percentages from 100% represent the % effect and is used in graphs such as fig. 6.

ations from 6.6% for the original suspension to 25% for some of the other counts. These deviations indicate a fairly high experimental error. Higher accuracy in the titration of the hormone would require a larger number of counts than was done in the present experiment.

Associated with the decrease in the number of viable cells was a corresponding increase in the stained cells. The control suspensions which were incubated 21 or more hours had many stained cells with granular, apparently well preserved nuclei (fig. 2). These cells were similar to those seen in the original suspension (fig. 1). Most of the stained cells, however, had pyknotic nuclei which were shrunken, round or oval and stained deeply and uniformly without any visible

logarithmic probability paper using the dosage as abscissae. The "effect" is represented on the ordinates and is calculated by subtracting the 50% survival time in per cent from 100%. It should be noted in passing that it is more convenient to indicate on the ordinate scale percentages, which have biologic significance, instead of probits, which are only mathematical abstracts. The dose-effect curve for 17-hydroxycorticosterone is shown in fig. 5.

The dosage producing the 50% effect may be termed the median effective dose. In other words the median effective dose (MED) is

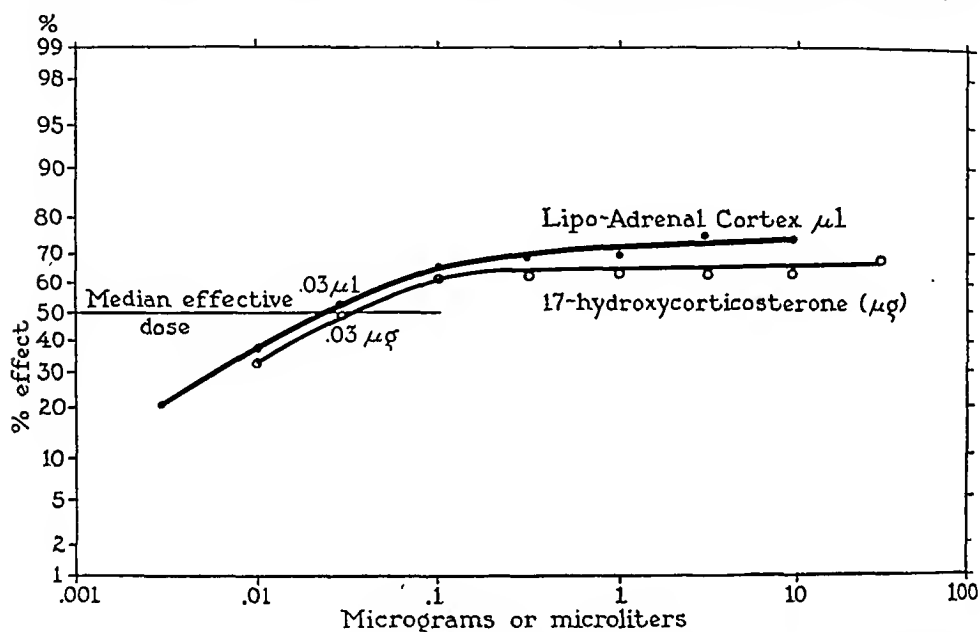


FIG. 5. The dose-effect curves on probit graph paper for thymic cells treated with Lipo-Adrenal Cortex and 17-hydroxycorticosterone. The dose-effect curves are curvilinear, show a maximum effect and permit the determination of a median effective dose.

that amount of reagent which reduces the 50% survival time of the cells by 50%. For 17-hydroxycorticosterone the MED was 0.03 microgram. The probable error of this constant cannot be determined by this graphical method, but it is undoubtedly very large due to an insufficient amount of data, and due to the low slope of the curve.

The dose-effect curve in fig. 5 is seen to be curvilinear and to approach a maximum effect of 70%. A dose as high as 30 micrograms (or about 1000 MED) failed to increase the effect beyond 70%. The minimum 50% survival time produced by the reagent was then about 9 hours.

The method described above for titration of 17-hydroxycorticosterone has, it is believed, theoretical significance and is particularly useful in studying a new compound. For practical purposes it is possible to simplify considerably both the experimental work and the calculations in determining the titration of the hormone. For this

method, it was necessary to consider only the viable cell counts at one particular time, as 21 hours. It is desirable to choose a standard observation period which should be approximately the 50% survival time of the cells studied (about 28 hours for cells of the rabbit thymus). The effect of the various amounts of reagent was then calculated using the viable cell count of the control (138 cells) as standard. A dose-effect curve was drawn on probit paper (fig. 6). This dose-effect curve is also curvilinear. Under these conditions a "standard effective dose" can be read from the graph and is found to be 0.02 microgram.

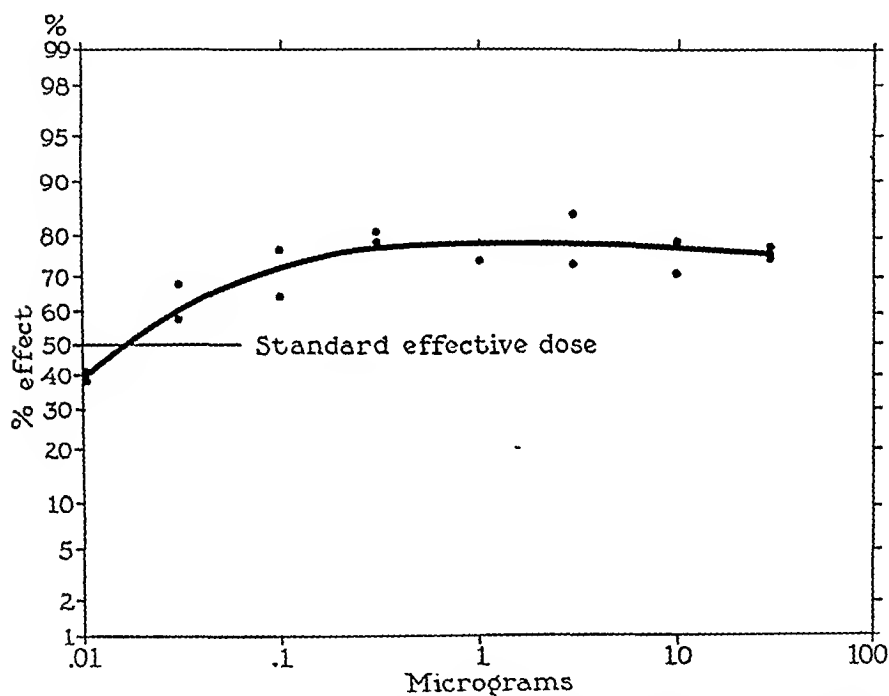


FIG. 6. Simplified dose-effect curve on probit graph paper for thymic cells treated with 17-hydroxycorticosterone and incubated for 22 hours at 37°C. As in Fig. 5, the dose-effect curve is curvilinear, has a maximum effect and permits the determination of a standard effective dose.

This compares with 0.03 microgram of the MED based on the 50% survival time.

Effect of hormones and steroids on lymphocytes. Dose-effect curves based on 50% survival time were also obtained for Lipo-Adrenal Cortex (fig. 5). This curve is also curvilinear and approaches a maximum of 70% effect. The MED is found to be 0.03 microliter or 0.001 rat unit according to the titration on the label of the commercial preparation. The dose-effect curves for Lipo-Adrenal Cortex and for 17-hydroxycorticosterone were similar in character.

TABLE 2. THE CYTOTOXICITY OF HORMONES AND EXTRACTS OF THE ADRENAL CORTEX AND OF NITROGEN MUSTARD AND X-RAYS TO CELLS OF THE RABBIT THYMUS. THE CYTOTOXICITY IS MEASURED BY THE MEDIAN EFFECTIVE DOSE OR THE DOSE THAT DECREASES THE 50% SURVIVAL TIME BY 50%

Reagent	Median effective dose	
	ml. or μ g.	Units
17-Hydroxycorticosterone	0.03 μ g.	
Corticosterone	1 μ g.	
Lipo-Adrenal Cortex	0.00,003 ml.	0.001 rat units
Eschatin	0.00,03 ml.	0.015 dog units
Extract of Adrenal Cortex	2.35 μ g.	0.002 rat units
Nitrogen mustard	0.46 μ g.	
X-rays		140 roentgens

Eschatin, an aqueous extract of the adrenal cortex had an MED of 0.0003 ml or 0.015 dog unit (table 2).

Several experiments on dry extract of the adrenal cortex gave an MED of 2.35 micrograms (table 2) which was equivalent to 0.002 rat unit according to the titrations obtained by Dr. Haines using the muscle glycogen method.

The crystalline steroid, corticosterone, produced the same dose-effect curve as Lipo-Adrenal Cortex. The MED was about 1.0 micrograms. It should be noted that all of the titrations are only approximate.

TABLE 3. THE LOW CYTOTOXICITY OF HORMONES TO CELLS OF THE THYMUS

Reagent	Dose in mg. producing		
	Moderate or 50% effect (median effective dose)	Slight effect	No appreciable effect
Desoxycorticosterone	—	0.5	0.05
Percorten (Ciba)			
Cortate (Shering)			
Adrenocorticotropin	—	0.2	0.1
Diovcylin (Ciba)	—	—	0.5
Di-ethylstilbestrol (Lilly)	0.25	0.03	—
Progesteron	—	1.0	0.25
Testosterone	0.2	—	—
Epinephrine	—	0.2	0.1

Desoxycorticosterone, in contrast, produced no effect even in a dosage of 0.05 milligram (table 3). The addition of 0.5 milligram had a slight toxic action which was less than a 50% effect.

The adrenocorticotropic hormone provided by Dr. Li produced little if any effect with a dosage of 0.2 milligrams. Other hormones were tested including diovcylin, diethylstilbesterol, progesteron, testosterone and epinephrine. All of these compounds, even in fairly high dosage, had little or no effect on the survival of cells derived from the rabbit thymus. In one experiment both adrenocorticotropic

hormone and Lipo-Adrenal Cortex were added to the suspension. It was found that the pituitary hormone did not increase or inhibit the activity of the Lipo-Adrenal Cortex.

Dose-effect curves for nitrogen mustard and x-rays. The peculiarities of the dose-effect curves for the steroids can be better understood by comparison with the curves for other reagents, such as nitrogen mustard and x-rays (fig. 7). The dosage on the abscissae of the graph is expressed in terms of MED which is about 140 r for x-rays and 0.46

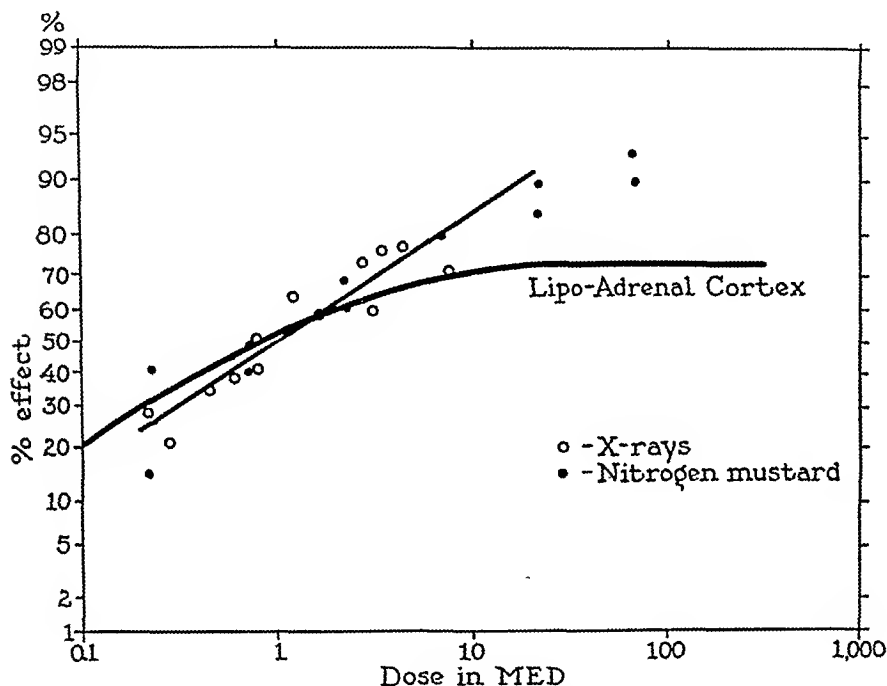


FIG. 7. A comparison between the dose-effect curve for Lipo-Adrenal Cortex, x-rays and nitrogen mustard. The dosage is given in terms of the median effective dose for each reagent. The dose-effect curves for x-rays and nitrogen mustard are identical, and differ from the dose-effect curve for Lipo-Adrenal Cortex in being linear and in not having a maximum effect.

microgram for nitrogen mustard. The figure shows that the dose effect curves for the two reagents are, to a considerable extent, linear and have approximately the same slope. About 93% effect was produced by 90 MED's of nitrogen mustard as compared to a 70% maximum effect by 300 MED's of Lipo-Adrenal Cortex.

From this comparison, it appears that the dose-effect curves of the adrenal steroids are characterized by the curvature, the 70% maximum effect and the low median effective dose. These three peculiarities in the curves seem dependent upon the nature of the toxic action produced by Lipo-Adrenal Cortex, Eschatin, corticosterone and 17-hydroxycorticosterone. From a teleologic point of view, it does

27°C. the maximum effect obtained was less than 50% so that one has to consider the MED is greater than the maximum dose tested or 1 rat unit. At 45°, the reagent had no appreciable effect on the survival of the cells. It would seem then that the Lipo-Adrenal Cortex affected the survival of the lymphocytes only when the cells were incubated at approximately physiologic temperature.

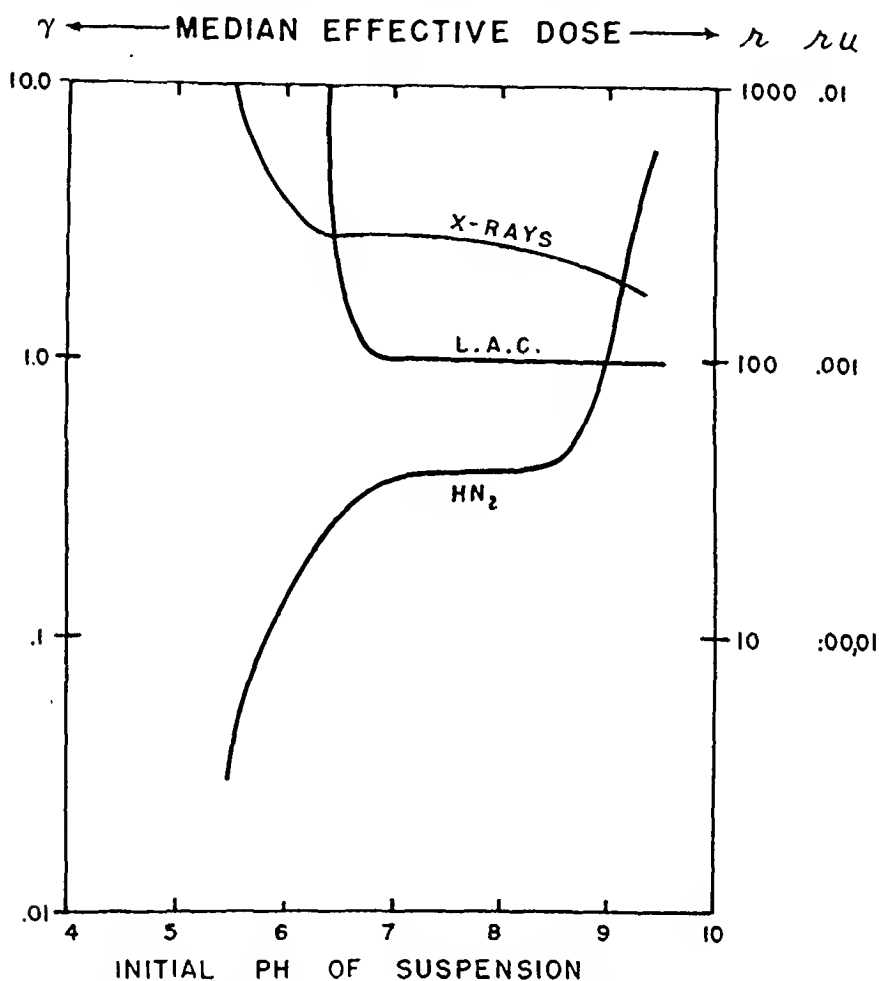


FIG. 10. The effect of the pH of the suspension on the median effective dose of Lipo-Adrenal Cortex, x-rays and nitrogen mustard for thymic cells. Acid reduced the susceptibility of the cells to the action of Lipo-Adrenal Cortex and x-rays but increased their susceptibility to nitrogen mustard.

It is of interest that x-rays also had a narrow temperature range of effectiveness as can be seen in the figure. At 27° and 41° the median effective dose of x-rays was less than at 37°, but at 17° and 45° x-rays had no appreciable effect on the survival of the cells.

Similar studies have been done with nitrogen mustard as a reagent. This reagent had a median effective dose of 0.46 microgram at 37°C. Varying the temperature between 17° and 45° had but little effect on the MED of nitrogen mustard. At 50°, however, the cells were resist-

ant to the action of nitrogen mustard and, in fact, the treated cells survived longer than the untreated ones. Nitrogen mustard then was toxic to cells over a wide range of temperature whereas x-rays and Lipo-Adrenal Cortex was effective only in a narrow range.

Effect of pH. A few experiments were done on the susceptibility of cells to Lipo-Adrenal Cortex, x-rays and nitrogen mustard, at different pHs. The pH of the suspension was altered by addition of hydrochloric acid or sodium hydroxide before Lipo-Adrenal Cortex or nitrogen mustard was added. In the case of the suspensions treated with x-rays, the cells were first radiated and then the pH of the suspension was adjusted. The susceptibility of the cells as measured by the median effective dose are represented graphically in fig. 10. It is seen that the cells in a fluid at a pH of approximately 6 were resistant to the action of both Lipo-Adrenal Cortex and x-rays, but they were relatively more susceptible to the action of nitrogen mustard. At pH of 9, the cells were approximately as sensitive to the action of x-rays and Lipo-Adrenal Cortex as at pH 7, but the toxicity of nitrogen mustard appeared to be increased in alkaline medium. There seems then to be a similarity in the effect of x-rays and Lipo-Adrenal Cortex on cells in acid and alkaline media.

DISCUSSION

Toxicity of hormones of the adrenal cortex. The present experiments showed that extracts and pure hormones of the adrenal cortex had an appreciable effect on cells of the rabbit thymus in vitro. This effect was evidenced by the increase in the number of cells which stained readily with safranin and by a decrease in the number of safranin-resistant cells. The findings were interpreted as indicating that the extracts and hormones caused a relative increase in the number of dead cells in the suspension and a decrease in the number of living cells.

The observations could be interpreted in three ways: 1) the steroids have a direct cytotoxic action on the lymphocytes; 2) they accelerate the normal rate of physiologic aging and death of the cells; or 3) they inhibit the mitotic activity and the growth of the lymphocytes in the suspension, but do not affect the death rate of the cells. No direct evidence has been obtained to support conclusively any one of these hypotheses. It seems, however, that the most plausible explanation of the observations is the second hypothesis that the steroids accelerate the aging and death of the cells.

The experimental findings indicate that the action of the steroids on lymphocytes had certain characteristics. First, the reagents tested had no immediate effect on the cells. Secondly, minute quantities of the steroids produced a detectable, delayed effect. Third, large doses had no greater effect than moderate doses. The maximum effect produced by the reagents was a relatively mild one. Finally the steroids

had no preceptible effect on intact dead lymphocytes in the suspension, nor on living cells of the bone marrow. These observations may aid in interpreting some of the reports in the literature.

In vivo, it has been shown by Dougherty and White (1945) and Selye (1947) that extracts of the adrenal cortex cause the necrosis of lymphoid tissue and ultimately the decrease in the weights of the lymphoid organs. The present in vitro findings are in accord with this in vivo observation of necrosis in lymphoid tissue. In vivo, the necrotic tissue would, of course, be rapidly absorbed whereas in vitro the dead cells remained intact.

The present findings are also in accord with the in vitro work of Heilman (1945). She reports that compound E, in concentrations of 1.25 micrograms per milliliter caused a moderate but significant inhibition of the migration of lymphocytes from explants of a rabbit lymph node in tissue culture. This inhibition was associated with an increased rate of degeneration of small and medium-sized lymphocytes in the migration zone. Increasing the concentration of the reagent failed to increase the degree of inhibition. In addition, Heilman (1945) found that the pure adrenal hormones had no inhibiting effect on macrophages in tissue culture. These findings are in agreement with the present observations of 1) the deleterious effect of pure adrenal steroids on rabbit lymphocytes, 2) the low dosage required for this effect, 3) the failure of larger doses to increase the effect and 4) the failure of the reagent to act on cells of the bone marrow.

Robertson (1948) observed that extracts of adrenal cortex failed to cause lysis of living lymphocytes in vitro. This finding is in agreement with the present observations that the steroids decreased the number of living and increased the number of dead cells, but had no appreciable effect on the total number of cells in the suspension.

Hechter and Stone (1948) found that extracts of adrenal cortex caused a rapid decrease in the number of lymphocytes in suspension. The apparent disagreement of this finding with the present observations may be attributed to differences in the experimental procedures.

It may not be amiss to emphasize that the observed toxic action of the steroids on lymphocytes is a relatively mild one. The method of testing has to be quite sensitive in order to demonstrate this activity of the hormones.

The mechanism of action of adrenal cortex hormones. The present findings throw some light on the problem of the mechanism. In the first place the end result of the action of the hormones on lymphocytes is a cell with a pyknotic nucleus. The same end result is obtained in cells undergoing spontaneous death and in cells killed with x-rays. Other toxic agents such as alcohol, heat, cold, formaldehyde and high concentrations of nitrogen mustard killed cells without producing a pyknotic nucleus.

There are several similarities in the reaction of the cells to x-rays

and to adrenal cortex hormones. The reagents were similar in that 1) they killed cells with the production of a pyknotic nucleus, 2) they were effective only when the cells were incubated in a narrow temperature range, 3) they were inhibited in the presence of acid, and 4) they were toxic to lymphocytes, but not to cells of bone marrow. The similarities would suggest that both reagents affect the same physiologic process within the cell. It would seem, furthermore, that both reagents accelerate the normal physiologic degeneration and death of the cells.

Current hypotheses suggest that x-rays affect the metabolism of thymonucleoprotein. There is then the possibility that the hormones of the adrenal cortex also affect the metabolism of this protein in lymphocytes.

In view of the similarity of the action of x-rays and extracts of adrenal cortex in lymphocytes, the question may be raised whether the two reagents affect other cells in similar manner. One action of x-rays is inhibition of growth. Recent studies have shown that hormones of the adrenal cortex can inhibit growth generally and locally. Another property of x-rays is inhibition of mitosis. Further investigation would be in order to determine whether adrenal cortex hormones have a direct effect on cells in mitotic division.

One difference in the behavior of cells treated with x-rays and with adrenal cortex hormones was observed in the present studies. The two reagents produced different dose-effect curves. For x-rays, the dose-effect curve was linear whereas for the hormones it was curvilinear. The curvilinear dose-effect curve for the hormones might be due to secondary factors such as permeability of the cell to the hormones.

SUMMARY

The following reagents had a delayed but not an immediate in vitro cytotoxic action on cells from the thymic gland of rabbits: Lipo-Adrenal Cortex, Eschatin, extracts of the adrenal cortex, corticosterone and 17-hydroxycorticosterone. These reagents were active in low concentrations. Increasing the dose of the reagent did not increase markedly the effect produced.

The Lipo-Adrenal Cortex was active when cells were incubated at 37° or 41°, but not at 27° or 45°.

Acid at a pH of 6 inhibited the action of Lipo-Adrenal Cortex.

No appreciable effect was observed with adrenocorticotrophic hormone, desoxycorticosterone and with various male and female sex hormones.

Lipo-Adrenal Cortex had no appreciable effect on cells of the bone marrow of rabbits.

There are several points of similarity in the action of x-rays and of Lipo-Adrenal Cortex on cells of the thymic gland.

The hypothesis is proposed that the extracts of the adrenal gland accelerate the normal degeneration and death of lymphocytes.

ACKNOWLEDGMENTS

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ADRENOTROPHIC ACTIVITY OF HUMAN BLOOD¹

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THE PRESENCE of adrenocorticotrophic hormone, or of a substance or substances possessing adrenotrophic activity, has not been convincingly demonstrated in human urine (Locke, Albert and Kepler, unpublished). We have therefore turned our attention to the blood, in the hope that adrenotrophic material may be present in a concentration sufficiently high to be measured by current assay procedures. A review of the literature pertinent to this subject is summarized in table 1. It appears that the results obtained are conflicting, and that much of this confusion has resulted from the use of test animals with intact pituitaries. Such a procedure does not, of course, permit specific detection of adrenotrophic activity in the material tested. Thus, there is considerable doubt as to the validity of all results obtained with human blood, with the sole exception of those obtained by Cooke, Graetzer and Reiss (1948).

The increasing emphasis on the study of pituitary-adrenal relationships makes evident the value of investigative work which may clarify this interrelationship. To avoid as far as possible the usual pitfalls encountered in studies of this type (Albert, 1949), we have investigated the problem of detection and assay of adrenotrophic hormone in human blood with the following points of emphasis: (1) the use of a sensitive and specific assay method so as to avoid spurious results, (2) the use of fresh untreated serum so as to obviate the use of empirical methods of concentration and purification, and (3) the use of blood from patients with adrenal disorders, situations in which extreme ranges of adrenotrophin might conceivably be encountered.

MATERIAL AND METHODS

The method of assay for adrenotrophic activity was that described by Sayers, Sayers and Woodbury (1948). Two minor modifications of this pro-

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TABLE 1. TESTS FOR ADRENOTROPIC ACTIVITY IN BLOOD (SERUM OR PLASMA)

Reference	Source of blood*	Preparation	Serum volume per animal, ml.	Assay method	Adrenotrophic activity
Jores (1935)	Cushing's syndrome	Deproteinized, concentrated serum	≈12	Increase in adrenal wt., and histologic change in intact mice	Yes
Sievert (1938)	Hypertensives 1. Benign 2. Malignant 3. Nephritic	Plasma ultrafiltrate	3-6	Disappearance of sudanophobe zone in intact mice	1. Yes 2. Yes 3. No
Paschkis (1940)	1. Cushing's syndrome 2. Adrenal cortical tumor 3. Hypertensives	Not stated	Not stated	Method of Jores (above)	1. Yes 2. No 3. No
Rakoff, Cantarow and Paschkis (1941)	Cushing's syndrome	Not stated	Not stated	Method of Jores	Yes
Cabeza (1942)	Diabetics	Deproteinized, heated and concentrated serum	≈20	Increase in adrenal wt., and histologic change in intact mice	Yes
Bartelheimer and Cabeza (1942)	1. Insulin hypoglycemia 2. Cushing's syndrome 3. Morgagni's syndrome 4. Acromegaly 5. Diabetics	Deproteinized, heated and concentrated serum	≈20	Method of Cabeza (above)	1. Yes 2. Yes 3. Yes 4. Yes 5. Yes
Golla and Reiss (1942)	Pregnant (mare)	1. Acetone dried serum 2. Boiled serum	Not stated	Increase in adrenal wt. in hypophysectomized rats	1. Yes 2. Yes
Faber (1945)	Adrenalectomized (rat)	Untreated serum	10	Increase in adrenal wt. and histologic change in hypophysectomized rats	No
Cooke, Gractzer and Reiss (1948)	Normal	Acetone precipitate of plasma	5-10	Ascorbic acid depletion in adrenals of hypophysectomized rats	Yes

* Except when otherwise indicated, all tests were performed with human blood.

cedure were employed: (1) hypophysectomized male rats of the Wistar strain were used as the test animals instead of the Sprague-Dawley strain used by Sayers and associates, and (2) the serum was administered via the inferior vena cava at the level of the right renal vein instead of via the tail vein.

Blood was obtained from normal subjects, patients with adrenal cortical hyperfunction and patients with adrenal cortical insufficiency by venipuncture in the antecubital fossa. In a few cases, blood was obtained from the external jugular vein. It was allowed to stand for two to four hours and then centrifuged to recover the serum. The serum was refrigerated at 4°C. until

TABLE 2. ASCORBIC ACID CONCENTRATIONS IN THE ADRENALS OF HYPOPHYSECTOMIZED RATS GIVEN PHYSIOLOGIC SALINE SOLUTION

No. of test	Volume of saline, ml.	Concentration of ascorbic acid, mg./100 gm. tissue		
		Left adrenal (before saline)	Right adrenal (1 hr. after saline)	Left-Right (L-R)
1	2.5	480.9	494.0	-13.1
2	3.0	457.1	467.6	-10.5
3	2.5	464.7	443.3	21.4
4	3.0	389.3	378.3	11.0
5	2.5	458.8	486.7	-27.9
6	2.5	496.1	516.1	-20.0
7	2.5	473.7	484.8	-11.1
8	2.5	516.2	512.2	4.0
9	2.5	507.8	522.5	-14.7
10	2.5	593.6	593.2	0.4
11	2.5	507.3	531.4	-24.1
12	2.5	488.7	491.6	- 2.9

Mean = $-7.3 \pm 4.2^*$

* Standard error of mean (Sem).

$$\text{Sem} = \frac{\text{S.D.}}{\sqrt{n}}$$

used, in most instances a period of about twenty hours. As a rule, 2.0 to 3.0 ml. of serum were injected into each rat, 3 rats being employed for each sample of serum. The determination of the ascorbic acid concentration of both adrenal glands was carried out according to the method described by Roe and Kuether (1943), adapted for use with tissue. Readings were made in a Coleman, Jr. spectrophotometer at a wavelength of 510 μ , and the ascorbic acid concentrations were reported in terms of milligrams of ascorbic acid per 100 gm. of fresh adrenal tissue.

Adrenotrophic activity was expressed as the difference in ascorbic acid concentration between the left adrenal, removed as a control before injection, and the right adrenal, removed one hour after the injection of the serum to be tested. This procedure is hereafter designated as "left minus right," or "L-R."

In order to obtain control values with which the experimental values could be compared, the effect of solutions containing no adrenotrophic activity on the adrenal ascorbic acid concentration was investigated. After the removal of the left adrenal, 2.5 to 3.0 ml. of 0.9 per cent saline solution were administered to 12 hypophysectomized rats (table 2) according to the previ-

ously described procedure. The concentration of ascorbic acid in the right gland one hour after administration of the solution was subtracted from the concentration of ascorbic acid in the left gland (L-R). In the group of 12 animals the mean concentration of ascorbic acid in the right gland was higher than in the left by 7.3 ± 4.2 mg. per 100 gm. of fresh tissue, or a mean L-R of -7.3 ± 4.2 . Although in principle these results are in agreement with those of Sayers, Sayers and Woodbury, our variation appears to be much greater than that encountered by these investigators. Our variability would indicate that no single L-R value less than 20 mg. can be accepted as definitely indicative of the presence of adrenotrophic activity, at least under the test conditions used by us.

RESULTS

Adrenotrophic Activity of Normal Serum.—Serum obtained from 3 normal women and 4 normal men, whose ages ranged from 18 to 45 years, was assayed for adrenotrophic activity, as shown in table 3. Only 2 of the 7 serums caused the ascorbic acid concentration of the right gland to fall below that of the left, so that the mean L-R was -4.4 ± 5.9 mg. However, serum obtained from the external jugular vein of 1 normal subject and administered to 3 rats in a dose of 1.0 ml. gave a mean L-R of 34.9 mg. The latter data are not included in table 3.

Adrenotrophic Activity of Serum in Patients Having Adrenal Cortical Hyperfunction.—Serum was obtained from 6 patients with adrenal cortical hyperfunction, proved at surgical exploration to be due

TABLE 3. ASSAY OF NORMAL SERUM* FOR ADRENOTROPHIC ACTIVITY

Patient, sex and age, years	Reduction of adrenal ascorbic acid, left gland-right gland (L-R), mg./100 gm. fresh adrenal tissue		
	Individual values		Mean
1 Woman 21	-11.4, -22.1, 21.1		- 4.1
2 Woman 45	-24.0, -13.8		-18.9
3 Woman 18	- 2.4, -53.5		-27.9
4 Man 38	37.9, 15.2, 0.4		17.8
5 Man 29	12.1, 2.6, 17.1		10.6
6 Man 28	-11.4, -22.9, 14.5		- 6.6
7 Man 33	21.3, - 0.9, -24.8		- 1.5

Group mean = -4.4 ± 5.9

* 2.5 ml. per test animal.

TABLE 4. ASSAY OF ADRENOTROPHIC ACTIVITY IN THE SERUM OF PATIENTS HAVING ADRENAL CORTICAL HYPERFUNCTION

Patient, sex and age, years	Diagnosis	Serum volume per test animal, ml.	Reduction of adrenal ascorbic acid, left gland-right gland (L-R), mg./100 gm. fresh adrenal tissue	
			Individual values	Mean
1 Woman 32	Cushing's syndrome. Adrenal cortical hyperplasia	2.0-3.0	31.1, 22.5	26.8
2 Woman 36	Cushing's syndrome. Adrenal cortical hyperplasia	2.1	10.7, -17.1	- 3.2
3 Woman 24	Cushing's syndrome. Adrenal cortical tumor	2.5	56.0, 36.1, - 5.0	29.0
3'	As above. 12 days after operation	2.5	10.6, 12.5, 44.6	22.6*
4 Woman 27	Virilizing adrenal tumor without Cushing's syndrome	2.5	9.1, -26.7, 18.1	0.2
5 Woman 25	Cushing's syndrome, persistent 5 mo. after subtotal resection of one hyperplastic adrenal	1.5	-31.4, -26.4	-28.9
6 Woman 32	Cushing's syndrome, 10 days after subtotal resection of one hyperplastic adrenal	2.5	16.9, -18.6, -28.8	-10.2*

Group mean = 4.8 ± 10.6

* These are not included in the group mean because of recent operative procedure.

either to tumor or to hyperplasia. All of the patients exhibited clinical and laboratory evidence of adrenal cortical hyperfunction at the time the blood was drawn, including those who were studied post-operatively. Two patients with hyperplasia (table 4, nos. 1 and 2), and 2 with tumor (nos. 3 and 4) had not had previous surgical treatment. One patient (no. 5) had persistence of Cushing's syndrome following subtotal resection of one hyperplastic adrenal gland five months previously. Blood from 1 patient (no. 6) with hyperplasia was taken ten days after the subtotal resection of one gland. (Values obtained from this patient, however, were not averaged with the others because of the recent major surgical procedure.) Also, blood was obtained twelve days after the surgical removal of a tumor from one of the patients whose blood was studied preoperatively as well (no. 3').

No consistent results were obtained from the serums of this group

TABLE 5. ASSAY OF ADRENOTROPHIC ACTIVITY IN THE SERUM OF PATIENTS HAVING ADRENAL CORTICAL INSUFFICIENCY

Patient, sex and age, years	Diagnosis	Serum volume per test animal, ml.	Reduction of adrenal ascorbic acid, left gland-right gland (L-R), mg./100 gm. fresh adrenal tissue	
			Individual values	Mean
Comparable cases of adrenal insufficiency				
1 Man 37	Addison's disease. Untreated	2.5	17.3, -23.8, 18.3	3.9
2 Man 41	Addison's disease. No treat- ment for 3 months	3.0	44.0, 15.8	29.9
3 Woman 54	Addison's disease. Untreated	2.5	8.7, 54.1, 18.4	27.1
4 Woman 59	Addison's disease. Untreated	2.5	13.3, 49.3, 7.6	23.4
5 Woman 65	Addison's disease. Untreated	2.5	31.8, 8.3, 39.4	26.5
Group mean = 22.2 ± 5.2				
Miscellaneous cases of adrenal insufficiency				
1 (see above)	Addison's disease. Untreated	3.0*	101.7, 36.8	69.2
6 Man 18	Addison's disease. Untreated	2.5*	44.7, 39.7	42.2
7 Woman 48	Addison's disease. (?) Un- treated. Elevated 17-keto- steroid excretion	2.5	20.3, 19.8, 25.3, -30.1, 9.9	9.0
8 Woman 27	Addison's disease. Off ad- renal cortical extract 48 hours	2.5	24.8, 1.1, 10.4	12.1
5 (see above)	Addison's disease after treat- ment for 1 week with DOCA 3 mg., testosterone 20 mg. and NaCl 5 gm., daily	2.5	1.1, -18.8	-8.8

* Represents use of serum from external jugular vein.

of patients. The serum from 1 patient with hyperplasia (no. 1), and 1 patient with tumor (no. 3), both of whom had Cushing's syndrome, caused a significant decrease in the ascorbic acid concentration of the right adrenal, but the other 4 serums gave negative results. It is interesting that the serum of patient no. 3 with cortical tumor had comparable positive values both preoperatively and postoperatively. The mean L-R of this group of patients, excluding nos. 6 and 3', was 4.8 ± 10.6 mg.

Adrenotrophic Activity in Serum of Patients Having Adrenal Insufficiency.—Eight patients with the diagnosis of Addison's disease were studied. All of their serums gave positive responses of varying magnitude (table 5). With the exception of patient no. 8 who had been without treatment for only 48 hours, all the others were previously untreated or had not received any therapy for a period of several months. The serum obtained from patients nos. 1 to 5 all caused a drop in the ascorbic acid concentrations of the right adrenal of the test animals. For these 5 cases the individual mean L-R varied from 3.9 to 29.9 mg. and the group mean was 22.2 ± 5.2 mg.

Serum from the external jugular vein secured from patients nos. 1 and 6 was found to cause mean decreases of 69.2 and 42.2 mg., respectively. The serum of patient no. 8, who had been without treatment for only 48 hours, had the relatively low mean L-R of 12.1 mg. A similarly low value of 9.0 mg. was obtained from the serum of patient no. 7 who had an unexplained high excretion of 17-ketosteroids despite the other criteria of adrenal insufficiency. It is also interesting that the serum obtained from patient no. 5 one week after adequate replacement therapy gave a mean L-R of -8.8 mg., compared to 26.5 mg. prior to treatment.

COMMENT

Since the ability of adrenotrophin to reduce the ascorbic acid concentration of the adrenals in hypophysectomized rats has been established as a distinct and specific reaction, a similar response following the injection of serum should be diagnostic of the presence of adrenotrophin in that serum. Our over-all results would indicate that adrenotrophic activity is present in the serum of patients with adrenal cortical insufficiency, and not present in the blood of normal persons or in patients with adrenal cortical hyperfunction. This statement, however, must be qualified in several respects.

The first qualification pertains to the small number of cases studied. However, the small number is perhaps pardonable when it is recalled that both untreated hypofunctioning and hyperfunctioning adrenal cortical states are extremely rare disorders. In spite of this drawback, we feel that the results obtained are indicative of a difference in these situations with respect to adrenotrophic content. Thus, serum from normal persons did not differ significantly from the saline controls ($t=0.34$, $p=0.7$). Neither did the serum of patients having adrenal cortical hyperfunction appear different, although the variability encountered in hyperfunctioning states makes statistical analysis untenable. In contrast, serum obtained from patients with adrenal insufficiency gave positive responses in all cases, being significantly elevated from the saline controls ($t=4.4$, $p<0.01$), and from normal serum ($t=3.3$, $p<0.01$). Thus there would appear to be significant amounts of adrenotrophic material in

the serum of patients with primary cortical hypofunction. This is in general agreement with the concept of an inverse relationship between adrenal function and adrenotrophic function of the pituitary, since all of our cases were considered to be primary adrenal insufficiency. This view is further supported by the studies on 1 patient with adrenal insufficiency whose serum contained adrenotrophic activity before treatment, but none after one week of adequate replacement therapy. The probability exists, however, that in adrenal hypofunction due to pituitary failure, adrenotrophic activity in the serum would be low to absent. Such cases, however, have not yet been studied.

The second qualification applies to the limited amount of test serum that we were able to administer to the individual rats. It is to be recalled that approximately 2.5 ml. of serum were used in all tests, since this was found to be the largest volume that could be injected without evidence of general toxicity and death. It is probable that adrenotrophic activity could be detected with a larger amount of serum, concentrated or purified in such a way as to render it nontoxic. Methods for accomplishing such a purification would be at best empiric until whole serum can be successfully assayed. Therefore, our results do not indicate the absence of adrenotrophin in the blood of normal and hyperfunctioning adrenal cortical states, but merely a concentration too low for 2.5 ml. of whole serum to produce a response under the conditions of the test.

The third qualification pertains to the results obtained in hyperfunctioning adrenal cortical states. The wide variability encountered obviates a decision as to the presence of adrenotrophin in these conditions. Some of the values would seem to indicate increased activity, but the mean of the group as a whole is not significantly different from that of normal serum. Here one may be dealing with two entities with respect to adrenotrophic activity. Hyperplasia may or may not represent a primary pituitary disorder with resultant increased adrenotrophic activity, but active cortical tumor probably represents a pure adrenal disease and as such should not be associated with elevated adrenotrophic activity. It is regretted, however, that our cases have been too few to permit us to draw accurate conclusions regarding the adrenotrophic content of the serum in adrenal cortical hyperfunction.

Apart from these qualifications, these experiments indicate that it is possible to detect adrenotrophic activity in the serum of patients having adrenal cortical insufficiency with the present assay method. With this as a basis, it should be possible to improve the method in the following ways: (1) reduction of the variability of response so as to make significant an L-R value of 10 mg. or less, (2) purification of serum so as to permit the administration of large volumes without toxicity, or (3) the use of jugular blood, which may contain a higher concentration of adrenotrophin than peripheral venous blood. With

such improvement, clear-cut levels of adrenotrophic activity in normal as well as pathologic states may be established.

SUMMARY

Adrenotrophic activity has been demonstrated in the blood of patients with adrenal cortical insufficiency. Conclusive demonstration of adrenotrophic activity in the blood of normal subjects and of patients with adrenal cortical hyperfunction was not achieved.

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NOTES AND COMMENTS

THE DIABETOGENIC EFFECT OF DEHYDROASCORBIC ACID

Of the known naturally occurring compounds, dehydroascorbic acid has a structure that most nearly approaches that of alloxan. The physical and chemical properties of these two compounds are strikingly similar. Dehydroascorbic is prepared by the oxidation of ascorbic acid with quinone (Moll and Wieters, 1936). It produces a characteristic excitement in rats with an LD50 equal to 320 mgs. per kilogram (120 gram rats). The surviving rats tolerate a second dose, three to four times as large as the first. The action of dehydroascorbic acid in producing a diabetogenic state is summarized in Table 1. The results are similar to those obtained following the injection of estrogens (Ingle, 1941; Ingle, Nezamis and Prestrud, 1947) and adrenal steroids (Ingle, 1941) in forced-fed rats, except that the effects are more pronounced and there is no need for special diets. A large dose of dehydroascorbic acid produces hyperglycemia lasting several days. Repeat doses after the blood sugar returns to normal again produces hyperglycemia. There is an increasing effect with increasing dose. Smaller doses injected daily produce hyperglycemia. Following a large dose of dehydroascorbic acid, there is the same triphasic hyperglycemia, hypoglycemia and hyperglycemia as is seen following alloxan injection. The possible position of dehydroascorbic acid as a mediating substance in the diabetogenic effects of estrogens and adrenal steroids, and the possible action of alloxan on an ascorbic acid-dehydroascorbic acid system in the beta cells are under further investigation.

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TABLE 1. EFFECT OF DEHYDROASCORBIC ACID ON BLOOD SUGAR

No. rats	Weight		Dose dehydro-ascorbic acid mg.	Average blood sugar mg./100 cc.									
	Range grams	Average grams		0hrs.	3 hrs.	8 hrs.	1 day	2 days	3 days	4 days	5 days	6 days	7 days.
4	116-120	118	40+130	110	120	125	110	125	330	182	156	132	130
2	115-118	117	20+80	144	121	98	229	150	117	118			
3	118-122	120	20+80	112	111	82	96	131	116	99			
5	116-120	119	20+60	138	125	114	123	176	112	116			
6	120-140	128	30	105			131	137	113	131			
5*	110-160	136	60 per day	123			122	201	247	232	173†	134†	141‡

* Same rats as in group 4 having received 60 mg. 8, 12 and 16 days previously, reinjected as shown.

† Four rats.

‡ Two rats.

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EXOPHTHALMOS IN RATS AFTER PROLONGED ADMINISTRATION OF PROPYLTHIOURACIL

Rats of a Wistar strain were kept on diets containing 0.02% propylthiouracil for many months. After about three months it was observed that some of the animals became exophthalmic. By the end of seven months almost every animal receiving propylthiouracil showed exophthalmos. Control rats on similar diets without propylthiouracil did not. The exophthalmos can be seen best when the animals are active and alert and are attentive to their surroundings. It may not be apparent at all when the eyes are closed in sleep or in anaesthesia. All animals receiving the drug are underweight com-

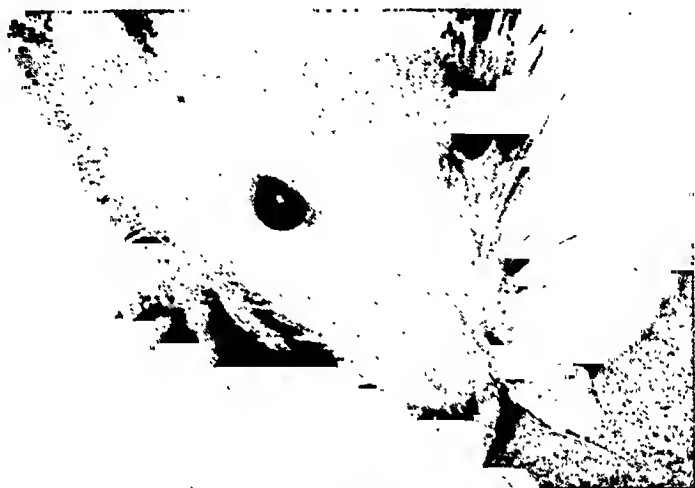


FIG 1. This rat received a diet containing 0.02 per cent propylthiouracil in the diet for a period of 22 weeks. Note the exophthalmos.

pared with controls and show typical myxedematous changes—scanty coarse hair, rough skin, and low metabolic rate (-30 to -40%).

While the exophthalmos is striking, its anatomical basis is not clear. In the animals autopsied up to the present, the weights of the eyeballs and of the orbital contents were not greater than in controls. The quantity of retrobulbar fat was negligible. The lymphocytic infiltration of retrobulbar tissues which has been described in Graves Disease (Salter and Soley 1944) was not found in these animals.

These observations do not entirely preclude the possibility of some edema of the orbital tissues but they do suggest that other factors such as pressure from the muscular floor of the orbit or abnormal retraction of the eyelids are more important.

One is tempted to draw a parallel between these observations and the exophthalmos which has been produced by the injection of thyrotropic hormone into guinea pigs and to the progressive exophthalmos which sometimes follows the treatment of Graves Disease by thiouracil derivatives (Williams and Bissell 1943; Haines and Keating 1946). It is not, however, our intention

to imply such parallelism unless further studies establish their anatomical or pathological similarity.

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TESTICULAR CHANGES IN THE JUVENILE PIGEON DUE TO PROGESTERONE TREATMENT¹

Nelson (1936) reported that progestin prevents involution of the testicular tubules in hypophysectomized rats. Selye (1940) observed atrophic changes in the testis of the same species after administration of progesterone. In a later paper, however, Selye and Friedman (1941) showed that progesterone caused only a slight atrophy of the interstitial tissue in mice and rats but the seminiferous epithelium remained undamaged. In mature doves results comparable to those reported by Selye and Friedman (1941) for rats and mice, have been obtained after treatment with progesterone (Lahr and Riddle, 1944). However, the action of this hormone on the testis of juvenile birds has not hitherto been reported.

In view of the theoretical interest in the effects of steroid hormones on the testis, it seemed desirable to contribute the present observations, since progesterone treatment was here found for the first time to produce a stimulating action on the juvenile bird testis.

PROCEDURE

Juvenile Indian Rock pigeons (*Columba livia intermedia* Strickl.) 60 days old were involved in this study. The initial body weight of each bird was taken at the commencement of the experiment and the final weight was noted on the day of autopsy. Progesterone was injected intra-muscularly into the breast muscles (0.5 mg. in 0.1 cc. of sesame oil daily) for a period of 30 days. The control birds were injected daily in an identical manner with 0.1 cc. of sesame oil alone for the same period.

All the birds were autopsied on the day following the last injections. The testes were carefully dissected out, weighed to the nearest mg., and finally fixed in alcoholic Bouin's fluid for histological studies. Serial sections of the testes were prepared by the paraffin method and stained with Heidenhain's iron-hematoxylin followed by eosin.

EXPERIMENTAL RESULTS

Gross examinations at autopsy revealed pronounced enlargement of the testis of the experimental males in contrast to that of the control birds. A

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¹ Grateful acknowledgment is made to Messrs. Ciba Pharma Ltd. of Bombay for the generous contribution of progesterone used in this study.

perusal of table 1 will also indicate that the testicular weights of the progesterone recipients were significantly greater as compared to those of the control specimens.

Microscopical examination of the testis of the control birds showed a compact appearance typical of immature testis. The tubular epithelium could be identified as a single layer of cuboidal cells. The lumen of the tubules was obliterated in the majority of the cases. The Leydig cells were sparsely distributed in the interstitium which was made up mostly of connective tissue cells.

Histological examination of the testis of the experimental males dis-

TABLE 1. TESTICULAR WEIGHTS IN CONTROL AND PROGESTERONE TREATED PIGEONS

	Number of birds	Testicular weight		Body weight at autopsy
		Absolute	Relative†	
		Mg.	Per cent	Gm.
Control	11	98.2 ± 1.5*	.0000041	203.2 ± 3.5
Progesterone treated	12	175.5 ± 2.1	.0000086	205.1 ± 3.8

† Organ weight expressed as percentage of body weight.

* Standard error of the mean.

closed a much less compact condition. The seminiferous tubules were enlarged in size and the tubular lumen appeared very conspicuous. Divisional stages were abundant in the tubular epithelium which presented a stratified appearance in contrast to its cuboidal condition in the control birds. The Leydig cells showed a marked predominance over the connective tissue cells in the interstitium. The former exhibited a peculiar tendency to form clusters of 3 to 4 cells.

DISCUSSION

Judging from the testis weights, treatment with progesterone significantly provoked testicular stimulation in the juvenile pigeons. The histological consequences of the enhanced testis weights in the treated birds were reflected in the enlargement of the seminiferous tubules and the marked increase in number of the Leydig cells.

A point of considerable interest was the conversion of the seminiferous epithelium from a cuboidal nature in control males to a stratified shape in the treated ones. If this finding is interpreted as an indicator of possible gametokinetic action of progesterone, then it becomes extremely important, since a substantial evidence is provided in favor of the stimulating action of a hormonally active steroid. Another significant item, and that is the marked increase in number of the Leydig cells in the treated males, should also be reckoned in this connection. This finding, therefore, appears to be at variance with the previous reports of Leydig cell atrophy (Selye, 1940; Selye and Friedman, 1941; Lahr and Riddle, 1944) due to progesterone treatment. Moreover, it provides a noteworthy exception to Selye and Albert's (1942) generalization that all hormonally active steroids injure the Leydig cells.

It has been satisfactorily established that the secretions of the anterior hypophysis must be regarded as the chief factors concerned with the regula-

tion of both the gametogenic and hormonal activities of the testis (*Vide* Turner, 1948). In the light of this knowledge, therefore, it can be assumed that in the present material progesterone treatment influenced the hypophysis to release greater amounts of ICSH and FSH which probably accounts for the increase in number of the Leydig cells as well as for the changes in the seminiferous epithelium.

SUMMARY

Intramuscular injections of progesterone (0.5 mg. daily for a period of 30 days) into 60 day old pigeons elicited significant increase in the testicular weights. Evidence is presented to show that the seminiferous epithelium was converted from a cuboidal state in the control males to a stratified shape in the hormone treated ones. There was also marked increase in number of the Leydig cells in the progesterone recipients. It is suggested that these changes were induced by the hormone through the augmentation of FSH and ICSH output of the anterior hypophysis.

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DECREASE OF THE SPREADING ACTION OF HYALURONIDASE IN PANCREOPRIVE DIABETES

The decreased resistance to infection in diabetes is a complex phenomenon, probably due to the following factors:

- a) Propagation of infection.
- b) Complement activity, bacteriostasis, phagocytosis, etc.
- c) Formation of antibodies.

On this hypothesis, we thought it would be interesting to find out the spreading action of hyaluronidase in experimental diabetes. Volterra and Giuli (1934) found a greater spreading action of hyaluronidase for trypan blue (1:300) in human diabetes.

MATERIAL AND METHODS

We used 12 dogs, both sexes, of medium weight (8-12 kgs.), hyaluronidase (Hydase) prepared by "Wyeth", and India ink "Pelikan, 541, Gunther Wagner" as the substance to be spread. Before pancreatectomy, in the normal animal, we explored the spreading of India ink by intradermic injection with the following solutions: A). 1 cc. saline solution 8.5% to which 21 drops India ink were added.—B). 1 mg. hyaluronidase ("Hydase") dissolved in

1 cc. saline solution 8.5% plus 21 drops India ink. The resulting surface was drawn on transparent cellophane paper and measured afterwards by a planigraph.

RESULTS

From Table 1, it is clearly concluded that removal of the pancreas produces a decrease in the spreading action of hyaluronidase in all the cases

TABLE 1. COMPARATIVE RESULTS OF THE SPREADING ACTION IN NORMAL DOGS THE SAME AFTER PANCRECTOMY, WITHOUT INSULIN CONTROL

Dog No.	Spreading action. MM^2		Differences
	Normal	Pancreoprive diabetes	
853	620	570	- 50
855	2,160	1,240	- 920
856	1,200	660	- 540
857	660	600	- 60
862	2,915	1,325	-1,590
865	1,535	1,250	- 285
866	2,225	750	-1,475
868	2,420	2,003	- 417
899	2,520	1,410	-1,110
900	1,900	1,550	- 350
901	3,020	1,240	-1,780
902	1,640	1,030	- 610

TABLE 2. COMPARATIVE RESULTS OF THE SPREADING ACTION IN NORMAL DOGS AND AFTER PANCRECTOMY. DIABETES CONTROLLED BY INSULIN

Dog No.	Spreading action. MM^2		Differences
	Normal	Controlled Pancreoprive diabetes	
865	1,535	2,740	+1,205
868	2,420	4,650	+2,230
899	2,520	1,560	- 960
900	1,900	2,890	+ 990
901	3,020	2,120	- 900
902	1,640	2,430	+ 790

studied. Results showed in Table 2 are less definite but very significant. In spite of the fact that there are only six cases, their statistical study clearly indicates a similar correlation.

SUMMARY

The spreading action of hyaluronidase in pancreoprive diabetes and the influence that insulin exerts on such a spreading action is studied. We have found that pancreoprive diabetes has an action which decreases the spreading effect. This fact is opposed to the result obtained by Volterra in human diabetes, in which he found a greater spreading action of hyaluronidase with regard to normal individuals.

ACKNOWLEDGMENT

We wish to express our best thanks to Dr. G. Angulo and "Wyeth International Corp." for generous shipments of "Hydase" and to Drs. Duran-Reynals and Seifter for helpful criticism.

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ASSOCIATION NOTICE

ANNOUNCEMENT

The membership roll of the Association is being revised in preparation for the issuance of a new Roster as of December 31, 1949. Copy for this must be in the hands of the printer not later than October 1, 1949.

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HENRY H. TURNER, M.D.
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IN VITRO EFFECT OF ADRENAL CORTICAL EXTRACT UPON LYMPHOCYTOLYSIS

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INTRODUCTION

LITTLE is known concerning the mechanism whereby adrenocortical hormones (ACH) produce lymphocyte dissolution *in vivo* (White and Dougherty, 1946). There is evidence, however, that adrenocortical steroids do not act directly upon lymphocytes to produce lysis. Thus, if has been reported that whole adrenal cortex extract (ACE) does not produce breakdown of the lymphocytes present in blood following *in vitro* incubation (Hechter, 1948) or of lymphocytes, obtained from lymph nodes, incubated *in vitro* in diluted homologous serum (Robertson, 1948). On the other hand, there is suggestive evidence that adrenocortical steroids accelerate *in vitro* lymphocyte breakdown provided that lymphoid tissue is present in the system. Thus, indirect evidence for increased lymphocyte dissolution has been obtained: (a) following addition of ACE, but not desoxycorticosterone acetate, to blood perfused through the isolated rabbit spleen (Hechter, 1948), and (b) in tissue culture experiments wherein 11-dehydrocorticosterone and 11-dehydro-17-hydroxycorticosterone increased the rate of degeneration of small and medium sized lymphocytes in the migration zone of lymph node implants (Heilman, 1945).

These *in vitro* studies taken together with the evidence that lymphoid tissue is the predominant site of lymphocyte breakdown *in situ* (White and Dougherty, 1946, Ehrlich, 1946), raise the possibility that the *in vivo* lymphocytolytic activity of ACH requires a factor present in lymphoid tissue. Studies were therefore undertaken to investigate the *in vitro* breakdown of lymphocytes in the presence of homogenates of lymphoid tissues and other organs and in other media both in the presence and absence of added steroids.

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METHODS

General: Lymphocyte suspensions obtained from the pooled lymphoid organs of rabbits were incubated with either tissue homogenates, homologous defibrinated blood or serum, or buffered salt solutions in the presence and absence of either ACE or crystalline steroids, using a clean but non-aseptic technic. Lymphocyte breakdown was evaluated by counting cells before and after various periods of incubation, usually one hour. To permit rapid differentiation of lymphocytes from spherical fat globules, and "debris" present in tissue homogenates, the cells were stained with brilliant-cresyl blue and counted under high dry magnification. Evidence will be presented later in this section of the paper that satisfactory cell counts are obtained with this procedure only under strictly defined conditions. Lymphocyte breakdown, as measured with this technic, thus refers to the disappearance of cells from the medium. Since the stainable cell structure observed is almost entirely nuclear, rupture of the cytoplasmic wall, unless simultaneously accompanied by nuclear dissolution, would not be measureable as lymphocytolysis. The evaluation of lymphocyte breakdown by a counting method requires that representative samples for cell counts be obtained from the incubation medium; thus, two separate counts were generally made to determine cell concentration and the sample was examined for cell agglutination or absorption upon particulate matter present in homogenates. These complications, however, were encountered but infrequently, and it would therefore appear that the lymphocyte disappearance observed was predominately due to lymphocyte breakdown.

It should be emphasized that the lymphocyte suspensions in the various experiments contain variable numbers of cells which are non-viable in that they are freely permeable to 1:1,000 eosin in Tyrode, in contrast to viable lymphocytes which are eosin-resistant (cf. Schrek, 1946a and 1946b); and finally, that the different suspensions contained variable percentages of lymphocytes derived from spleen, thymus and lymph nodes.

Experimental Animals: Rabbits, 3-5 months old, of various strains, weighing between 2.5 and 4.0 kg. were used. Marked variations were observed from animal to animal with respect to the individual weights of thymus, lymph nodes and spleen and in the total number of lymphocytes obtainable from the combined lymphoid organs studied. The latter variations could not be correlated to the weight of pooled lymphoid tissues or to the body weight of the rabbit.

Lymphocyte Suspensions: After the animal was killed by exsanguination, the spleen, thymus and cervical lymph nodes were removed, trimmed of fat and connective tissue, and weighed. The organs were minced with scissors in the presence of 10 to 15 cc. of either 0.9 per cent NaCl (hereafter referred to as saline) or Tyrode solution (adjusted to pH 7.4) and filtered through 4-6 layers of muslin. Excluding erythrocytes, practically all of the cells in these suspensions are lymphocytes and of these 75-85 per cent were small, 6-8 per cent large, and the remainder were classed as medium-sized as determined on air-dried smears stained with Wright's stain. The suspensions were prepared in the cold using chilled solutions and stored in the refrigerator until the experiment was initiated (usually 1-2 hours later). Those cell suspensions which were not treated further are contaminated with lymphoid tissue components, and will hereafter be referred to as "non-washed" cells. In ex-

periments where it was desired to remove the bulk of these contaminants the cells were separated by slow speed centrifugation (15 minutes at 1,000 RPM) in a cold room; the supernatant was discarded and replaced with fresh salt solution. A small amount of stringy material which does not go into the suspension was removed by filtration through muslin. Such cell suspensions even though they may be contaminated to a degree will be referred to as "washed" lymphocytes.

In other experiments, lymphocyte suspensions were prepared by direct homogenization of the lymphoid tissues in isotonic or near isotonic salt solutions using a "worm" Potter and Elvehjem (1936) homogenizer. The lymphoid tissue was directly homogenized with saline or more frequently with a solution containing equal parts of saline-phosphate buffer (pH 7.00 or 7.38) using 1.5 cc. solution per gram of tissue. The lymphocytes in these preparations (after staining air-dried smears with Wright's stain) are similar in appearance to stained preparations obtained by saline or Tyrode extraction of minced lymphoid tissue. However, these cells, in contrast to those obtained with saline, are uniformly freely permeable to eosin and thus may be assumed to be "non-viable." The lymphocytes in such homogenized suspensions will therefore be designated as "damaged" lymphocytes.

Homogenates: The lymphoid residue, remaining on the muslin after the lymphocyte suspension had been filtered, furnished the material for the lymphoid homogenate. The homogenates were prepared in the following manner: 1 part of tissue residue was homogenized with 1.5 parts of either iced distilled water or a solution consisting of equal parts of 1 per cent sodium chloride and M/15 phosphate buffer (pH 7.0) in the apparatus of Potter and Elvehjem. The homogenizing tube was held in ice water during the course of homogenization, and grinding was performed at intermittent intervals of 30-45 seconds. Upon completion of homogenization, the material was filtered through cheese cloth and 0.5 cc M/15 phosphate buffer (pH 7.0) was added to every 1 cc. of the water homogenate. Where homogenates were prepared with salt-phosphate solution instead of water, no further additions were made.

The number of cells in these lymphoid homogenates relative to the concentration of lymphocytes used in any experiment was so small (less than 3 per cent) that for practical purposes, these homogenates were regarded as "cell free."

Homogenates of brain and muscle were prepared similarly, except that these tissues were not extracted with salt solutions prior to homogenization. Homogenates of brain and muscle contain only a few cells which stain with cresyl-blue.

Steroids Investigated: The ACE utilized in these studies was a dried neutral ethyl acetate residue of hog adrenals furnished by Dr. M. Kuizenga of the Upjohn Company. The preparation contained 1.06 Ingle Work Units per mg. In addition to the ACE preparation, the following crystalline steroids were studied: corticosterone, 11-dehydrocorticosterone (Compound A of Kendall), 11-dehydro-17 hydroxycorticosterone (Compound E of Kendall), 11-desoxycorticosterone, 11-desoxycorticosterone acetate, α estradiol, ethyl testosterone, 17-hydroxyprogesterone, androsterone and cholesterol. Solutions of these steroids and ACE were prepared using redistilled acetone as solvent. Aliquot portions were added to test tubes (14×85 mm.) in which

the lymphocyte incubation studies were subsequently to be performed; the solvent was removed *in vacuo* and the tubes containing dried steroid were stored at 2° C. until used for experiments.

Evaluation of Lymphocyte Breakdown: Lymphocyte suspensions were pipetted into the tubes containing the dried steroids, and then an equal volume of either serum, blood, saline (buffered to pH 7.0 with M/150 phosphate or borate) or tissue homogenate added. (Where the "damaged" lymphocytes containing homogenized lymphoid tissues were tested, only the single suspension was employed.) The tubes were stoppered, mixed and samples were withdrawn to determine the initial cell concentration. Tubes were inserted in a machine which shook them horizontally 230 times a minute at 38°C. To insure thorough mixing a glass rod was added to each tube. The experiments were so designed that two tubes, containing no steroid, were controls for ACE or the other steroids investigated.

Samples from the incubation tubes were removed with either red or white blood cell pipettes, and a solution of 0.172 per cent brilliant cresyl-blue (C.I. No. 877, Nat. Aniline Div.) containing 0.04 per cent sodium cyanide was used as a diluting fluid. Cresyl-blue cyanide stains leucocytes blue, and hemolyzes erythrocytes. The cells were diluted either 1:10, 1:20, 1:100 or 1:200, so that at least 200 cells could be counted on 4 mm.² of the hemocytometer chamber. The pipette was gently shaken with a rotary motion for 3 minutes in a standardized manner. Only a single counting chamber was used in these experiments, and all pipetting and counting of cells were made by a single observer using a code system, so that each sample was an unknown.

Comparative studies using brilliant cresyl-blue cyanide and 1 per cent acetic acid as diluents on whole heparinized rabbit blood revealed that the total leucocyte count, with both diluents was almost identical. It was further demonstrated (using phosphate buffers) that variations of pH between 6.0 and 8.0 in the samples did not affect the capacity of the brilliant cresyl-blue cyanide to stain lymphocytes. Shifts in the pH of the incubation medium containing lymphocytes, therefore, could not give rise to false results on the basis that lymphocytes failed to take the stain at particular pH values.

Early in the course of this work it was discovered that significant lysis of of the cresyl-blue stained cells may occur in the blood pipette on standing at room temperature, and that the degree of this lysis was a function both of temperature and time. The error introduced as the result of this lysis, however, could be removed by the following procedure which was therefore uniformly employed: The incubated tubes were removed from the incubator and immediately chilled in an ice bath. Samples were obtained from these iced tubes after mixing; the cells were then diluted with cold cresyl-blue and the blood cell pipette containing the diluted suspension was then placed in the refrigerator at 2° until the cell count was actually made. Where lymphocyte breakdown was followed over a course of several hours, the tubes containing the incubated cells were not chilled, otherwise the remainder of the procedure described above was followed. The duplicability of counts taken under the above conditions is shown by the fact that when counts were made on two samples containing the identical constituents, in 100 consecutive cases the average percentage differences between the counts was 3.0 per cent (± 0.40 , standard error) despite the fact that one sample was counted 30–40

minutes after the other. This difference, however, is not the "error" of a single count; from the studies of Berkson et al. (1940) who have evaluated the errors involved in cell counts with a hemocytometer, it may be calculated that a single count, where 200 cells are counted, can be determined significantly only within about ± 20 per cent.

Counts on Eosin-Resistant and Eosin-Stainable Cells: To obtain information concerning the percentage of "eosin-resistant" and "eosin-stainable" cells in a given sample, separate counts were made using brilliant cresyl-blue and eosin 1:1000 in Tyrode (pH 7.4) as diluents. The total lymphocyte concentration was obtained from the count of cresyl-blue stained cells; the eosin-stained cells were next counted directly and the eosin-resistant cells were obtained by difference.

Lymphocytolysis in the Absence of Added Steroids

Before starting to examine the effect of ACE or other steroids, studies were undertaken to evaluate lymphocyte breakdown in the absence of added steroids. A summary of these studies, as they pertain to the subject of this communication, will be presented; full details will be reported elsewhere.

Lymphocyte suspensions undergo spontaneous breakdown upon storage in Tyrode or saline solution and upon incubation at 38° in serum or tissue homogenates. Freshly prepared cell suspensions are most stable in the cold (2° C.) and at slightly acidic pH's; elevation of temperature to 25° or 38° and pH to 7.4 increases the rate of spontaneous breakdown of cells. The rate of lymphocytolysis of cell suspensions stored in the cold is independent of the initial cell concentration (range 377,000 to 82,000 lymphocytes per mm.³) and is not directly related to the percentage of eosin-stainable cells initially present in the suspension. It appears likely that eosin-resistant cells are transformed to eosin-stainable cells prior to dissolution, and that the percentage of non-viable eosin-stainable cells in a suspension at a particular time is the resultant of the rates of conversion of eosin-resistant to eosin-stainable cells and the dissolution of the latter cells.

Upon incubating "washed" and "non-washed" lymphocyte suspensions in defibrinated blood, serum or tissue homogenates at 38° C., lymphocytolysis proceeds at a significant rate in the absence of steroids; consequently any effects of ACE or other steroids to be described later is to *alter the rate of lymphocytolysis and not to initiate this process*. It was uniformly observed that the degree of "spontaneous" lymphocytolysis during incubation was greatest with lymphoid homogenate and least with serum; lymphocyte breakdown in brain and muscle homogenates was somewhat greater than in serum but did not approach the rate observed with lymphoid homogenates. Lymphocytolysis of cells added to defibrinated blood is approximately equivalent to that observed in serum. Lymphoid homogenates, in addition to increasing the rate of lymphocytolysis, exhibit other evidences of cytotoxicity. Thus, lymphocytes incubated in lymphoid

homogenates noticeably decrease in cell volume after one hour incubation, and after 2.5 hours, in some cases, the cell size has diminished to such an extent that it is not possible to count the cells accurately using our usual magnification ($\times 400$). On the other hand, those lymphocytes incubated for 3 hours in Tyrode, whole blood, serum, or brain and muscle homogenates which have not undergone dissolution, retain their initial size or, if anything, tend to swell. In addition, lymphoid homogenates rapidly increase the permeability of lymphocytes to eosin so that soon after incubation has been initiated almost all of the lymphocytes present in the medium are "eosin-stainable". This effect upon the cytoplasmic permeability of lymphocytes is more or less specific for lymphoid homogenates and is not observed with Tyrode, serum or whole blood and only occurs to a limited extent with homogenates of brain and muscle.

The process of washing lymphocytes (*i.e.* centrifugation, etc.) does not significantly influence the stability or permeability of lymphocytes. However, the so-called "damaged" lymphocytes are more labile than the "washed" or "non-washed" lymphocyte suspensions and breakdown more rapidly (about 50%) than either "washed" or "non-washed" lymphocytes in lymphoid homogenates.

Considerable variation in the degree of lymphocyte breakdown was observed upon incubating cells in serum or tissue homogenates in the individual experiments under apparently identical conditions. The variations encountered may be due to the fact that (a) the lymphocyte suspensions were obtained from rabbits, heterogenous in age, weight and strain; (b) the cell suspensions were stored, in the cold, for various periods of time; and (c) the media employed for incubation were obtained from different animals. Of these factors (a) seems the most important since the marked variations persisted when lymphocytes from different animals were studied in a single medium (serum) under identical conditions.

Lymphocytolytic Index

In preliminary testing, it was noted that while ACE almost regularly increased the rate of cytolysis of cells incubated in lymphoid homogenates relative to the non-steroid control, the differences produced by ACE were small relative to the marked variation in the rate of lymphocytolysis in the non-steroid controls. Accordingly a lymphocytolytic index was devised, which is independent of the rate of lymphocyte breakdown in the control. For this purpose, the difference in cell count after incubation (one hour) between the treated sample and the non-steroid control (" Δ ") was determined. From this Δ , the difference due to treatment per 100 cells of the control sample (henceforth referred to as $\Delta\%$) was calculated. For example, if after one hour of incubation the cell concentration per mm.³ is 200×500 in the control and 160×500 in the ACE-treated sample the dif-

ference due to ACE at one hour (ΔACE) is -40×500 and the $\Delta\%$ is $\frac{-40 \times 500}{200 \times 500} \times 100$ or -20 . Using the $\Delta\%$ index, the non-steroid ACE control is automatically zero and increased cell breakdown is expressed as a negative $\Delta\%$ value, while inhibition of lymphocytolysis yields positive values. To ascertain the statistical significance of $\Delta\%$ values obtained in the presence of hormonal steroids, it was necessary to have information concerning the error of the method. This was determined by evaluating the $\Delta\%$ value of an inert steroid, cholesterol,

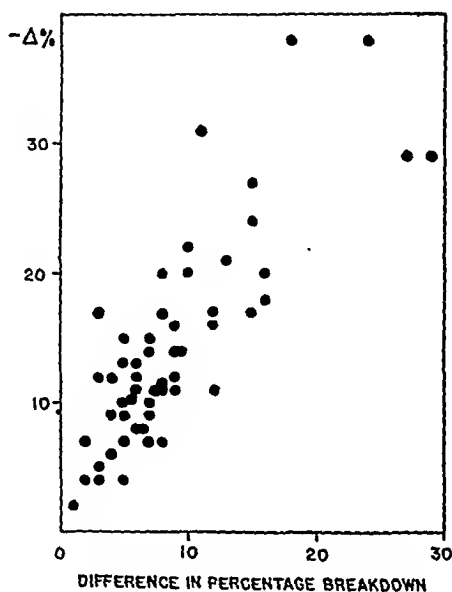


FIG. 1 illustrates the relationship between $\Delta\%$ and the difference in percentage breakdown induced by ACE ($10 \mu\text{g. per cc.}$) upon varying types of lymphocytes incubated in lymphoid homogenate for one hour at 38°C. Each dot represents an individual experiment.

arbitrarily tested at a concentration of $10 \mu\text{g. per cc.}$ under varying conditions. Under all conditions it was observed that $\Delta\%$ values obtained with cholesterol are not significantly different from zero, as evaluated by the null method. Consequently the significance of $\Delta\%$ values obtained with ACE or other steroids was determined by comparing the results obtained with those of the appropriate cholesterol group using the "t" test of Fisher (1936).

One may also evaluate lymphocyte breakdown by determining the difference in percentage lymphocyte breakdown in a given interval in the absence and presence of a steroid. However, in contrast to the $\Delta\%$ index, the values obtained with this procedure are influenced by the degree of spontaneous lymphocyte breakdown and tend to be

low when the latter factor is high. Figure 1 illustrates the relationship between $\Delta\%$ ACE and the difference in percentage breakdown produced by ACE in experiments where lymphocytes were incubated in lymphoid homogenates for one hour. It will be seen, in general, that $\Delta\%$ ACE is proportional to the difference in percentage breakdown produced by ACE; those points which deviate from the linear curve at high $\Delta\%$ values are experiments wherein a high degree of spontaneous breakdown was obtained.

Effect of ACE

In preliminary experiments, where ACE was incubated at 38° with lymphocytes in the presence of lymphoid homogenates for several hours, the lymphocytolytic activity of ACE tended to be most pronounced at the first hour of incubation. This finding, combined with the previously described observation of lymphocyte shrink-

TABLE 1. THE EFFECT OF ACE CONCENTRATION UPON THE BREAKDOWN OF "NON-WASHED" LYMPHOCYTES INCUBATED WITH LYMPHOID HOMOGENATES AT 38° FOR ONE HOUR

Steroid	Concentration	Number experiments	Mean $\Delta\%$	p†
ACE	<i>μg. per cc.</i>			
	1	5	$-5.2 \pm 1.8^*$	0.02-0.05
	5	5	-8.2 ± 2.1	<0.01
	10	5	-13.0 ± 1.8	<0.01
	50	5	-13.4 ± 2.2	<0.01
	100	5	-8.4 ± 2.0	<0.01
Cholesterol	10	8	-1.5 ± 1.3	—

* Standard error of the mean.

† P value as compared to the cholesterol mean; when "p" is less than 0.05, the difference is regarded as statistically significant.

age in lymphoid homogenates, led us to evaluate the lymphocytolytic activity of ACE (and other steroids studied) after an arbitrary period of one hour of incubation at 38° C.

Effect of ACE Upon Cells Incubated in Lymphoid Homogenate: In Table I data are presented illustrating the effect of ACE concentration upon lymphocytes incubated with homogenates of lymphoid tissue for 1 hour. In each of these 5 experiments "non-washed" lymphocytes from a single suspension were incubated with equal volumes of a single homogenate for one hour and the ACE concentration was varied from 1 to 100 μg per cc. To assess the significance of the ACE effects, the collected data of other experiments where cholesterol (10 μg . per cc.) was tested under similar conditions (*i.e.* "non-washed" lymphocytes incubated in lymphoid homogenates) is also shown in Table I. It will be seen that maximal lymphocytolytic activity as evaluated by the $\Delta\%$ index is obtained with the 10 μg . cc. dose. The significance of the drop in lymphocytolytic activity

noted when the ACE concentration is increased from 10 to 100 $\mu\text{g. per cc.}$ remains to be determined; it should, however, be noted that the difference in response between the 10 and 100 $\mu\text{g. per cc.}$ doses of ACE is not significant, in a statistical sense, and that cholesterol was not tested at a level of 100 $\mu\text{g. per cc.}$

Figure 2 shows a comparison of the lymphocytolytic activity of ACE upon "washed", "non-washed" and "damaged" suspensions incubated in the presence of lymphoid homogenate. In these experiments a 10 $\mu\text{g. per cc.}$ ACE dosage was employed, and cholesterol

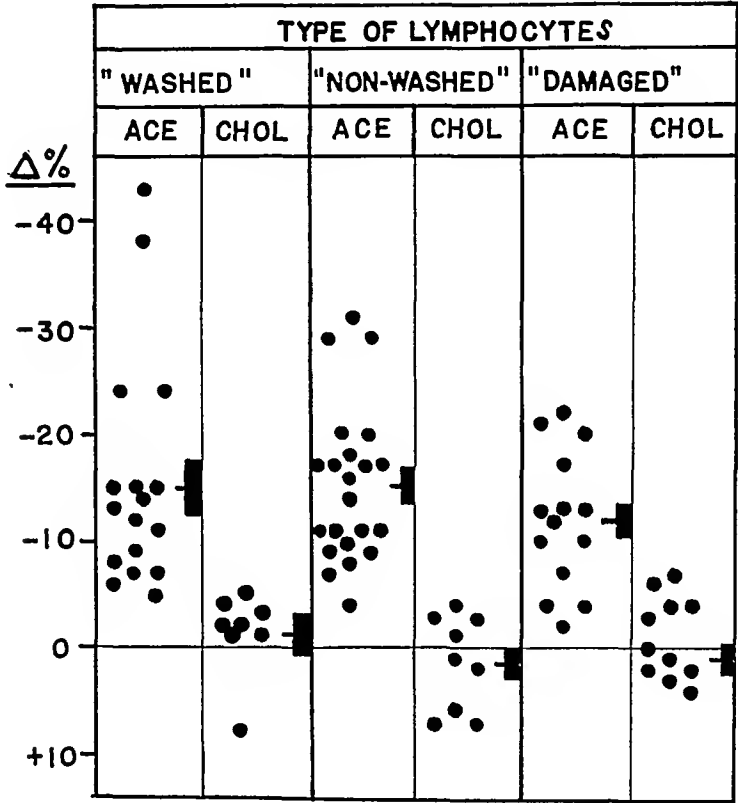


FIG. 2 illustrates the lymphocytolytic effect of 10 $\mu\text{g. per cc.}$ ACE and cholesterol (CHOL) upon varying types of lymphocytes incubated for one hour at 38° C. in lymphoid homogenates. Each dot represents a single experiment; the mean of each group is shown at the line projecting from the black bar; the standard error of the mean in each group is represented by the height of the black bar.

(10 $\mu\text{g. per cc.}$) was used as a control. It will be seen that in the presence of lymphoid homogenates, ACE significantly accelerates the breakdown of all types of lymphocyte suspensions.

To determine whether the $\Delta\%$ values obtained with ACE are influenced by factors such as the initial cell concentration, the lymphocyte concentration of the non-steroid control after 1 hour of

incubation and the degree of spontaneous breakdown, the individual ACE values were plotted against each of these variables and these relationships are illustrated in Figure 3. It will first be seen by the use of the $\Delta\%$ index that the lymphocytolytic activity of ACE is not significantly influenced by variations in the rate of spontaneous lymphocyte breakdown. There may be a tendency for $\Delta\%$ values produced by ACE to decline as the number of lymphocytes in the system increases. This is most evident when $\Delta\%$ ACE is plotted against log cell concentration of the control after 1 hour of incubation, and is less marked when plotted against the log of initial concentration.

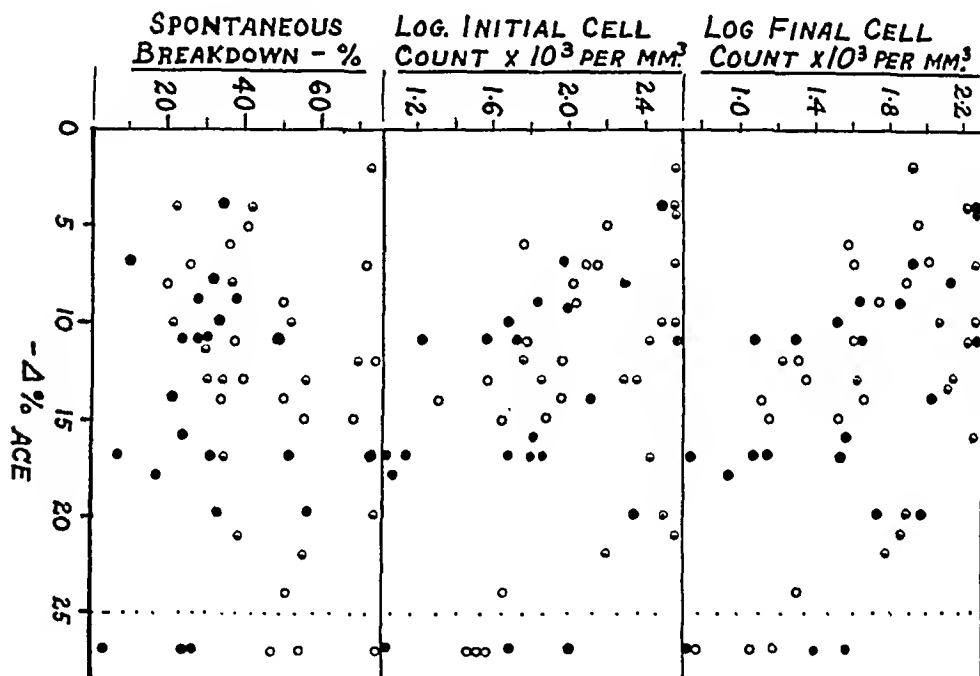


FIG. 3 illustrates the influence of spontaneous breakdown, the initial lymphocyte concentration, and the lymphocyte concentration in the control after one hour incubation (referred to as final cell concentration) upon $\Delta\%$ values obtained with ACE. Each circle represents an individual experiment wherein lymphocytes were incubated with lymphoid homogenate and ACE (10 $\mu\text{g. per cc.}$) for one hour at 38°C. The open circles (o) refer to the experiments wherein "washed" lymphocytes were employed; the filled-in circles (●) refer to "non-washed" cells, while the half-filled circles (◐) refer to the experiments using "damaged" lymphocytes. All $\Delta\%$ values greater than -25 are plotted at the right of the dotted line at an arbitrary $\Delta\%$ value of -28 .

Comparison of ACE lymphocytolytic activity in various media: In the next experiments, the effect of ACE upon cells incubated in media not containing lymphoid tissue constituents was tested. In seven experiments "washed" lymphocytes from a single suspension were added to serum, and to homogenates of brain, muscle and lymphoid tissue. (In each case the homogenate represented an equivalent amount of tissue from the same rabbit.) Lymphocytolysis in the

presence and absence of ACE (10 μ g. per cc.) in the various media was determined simultaneously. These results are shown in Table 2. In other studies, where ACE was tested upon lymphocytes incubated in whole blood, serum or buffered saline (pH 7.0), most of the experiments were not run concurrently with lymphoid homogenates. Thus, the composite results of these experiments are presented in Table 3.

The results shown in Tables 2 and 3 demonstrate the ACE, which is an effective lymphocytolytic agent in the presence of lymphoid homogenates, is without significant effect upon lymphocytes incubated in muscle homogenate, blood, serum or buffered saline. With

TABLE 2. COMPARATIVE LYTIC ACTIVITY OF ACE UPON "WASHED" LYMPHOCYTES INCUBATED IN SERUM AND TISSUE HOMOGENATES FOR ONE HOUR

Experiment Number	Initial Count $\times 10^3$	Serum		Lymphoid homogenate		Brain homogenate		Muscle homogenate	
		S.B.*	$\Delta\%$ ACE	S.B.	$\Delta\%$ ACE	S.B.	$\Delta\%$ ACE	S.B.	$\Delta\%$ ACE
	<i>cells per mm.³</i>	<i>per cent</i>		<i>per cent</i>		<i>per cent</i>		<i>per cent</i>	
1	30	0	-15	82	-38	0	-18	20	-6
2	25	34	+10	53	-43	32	-16	24	-7
3	130.5	27	+7	71	-7	36	-11	49	+14
4	108	16	-13	50	-9	26	-21	19	-4
5	150	39	+21	41	-5	26	-11	49	+4
6	57.5	11	+6	36	-6	22	-7	41	+2
7	76	31	-4	55	-15	36	-10	25	-4
Mean		22.6	+1.7	55.5	-17.6	25.4	-13.4	32.5	-0.1

* Spontaneous breakdown in the medium in the absence of steroids.

brain homogenates, as with lymphoid homogenates, ACE is active. It may be mentioned that Table 2 also demonstrates that ACE lymphocytolytic activity need not necessarily be associated with a high rate of spontaneous lymphocyte breakdown (as occurs with lymphoid homogenates) since the degree of spontaneous lymphocyte breakdown in homogenates of brain is less than that which occurs in muscle homogenates.

Nature of the Factor in Lymphoid Tissues

The differences in the lymphocytolytic activity of ACE observed with salt solutions, serum, whole blood and tissue homogenates seem best explained on the basis that homogenates of lymphoid organs and brain contain a factor which potentiates the lytic activity of ACE upon lymphocytes. Since lymphoid tissue is presumed to be the predominant site of lymphocyte breakdown *in situ*, it was of interest to obtain information concerning the nature of the factor present in lymphoid homogenates.

Thermolability of the Factor. The effect of heat treatment upon the ability of lymphoid homogenates to potentiate ACE lymphocyto-

TABLE 3. LYTIC ACTIVITY OF ACE UPON "WASHED" LYMPHOCYTES INCUBATED IN SERUM, BLOOD AND SALINE SOLUTIONS AS COMPARED TO LYMPHOID HOMOGENATES†

Incubation media	Steroid†	Number of experiments	Mean $\Delta\%$
Lymphoid Homogenate	ACE	17	$-15.0 \pm 2.7^*$
	CHOL.	10	-0.3 ± 1.4
Serum	ACE	14	-2.0 ± 2.6
	CHOL.	8	-0.1 ± 1.4
Buffered Saline	ACE	9	$+0.1 \pm 1.7$
	CHOL.	10	-0.9 ± 1.6
Blood	ACE	7	-2.3 ± 2.0
	CHOL.	6	-1.0 ± 1.8

* Standard error of the mean.

† Steroid concentration, 10 $\mu\text{g. per cc.}$

‡ Incubated for one hour at 38° C.

lytic activity was studied in five experiments. In each of these experiments a single "washed" lymphocyte suspension and homogenate of lymphoid tissue was employed. One portion of the homogenate was heated for 15 minutes in a steam bath; the other portion was not heated. Equal volumes of a cell suspension were then added to both types of homogenates, and the mixtures were incubated for 1 hour at 38° C., with and without ACE (10 $\mu\text{g. per cc.}$). The results of these experiments are illustrated in Table 4. It will be seen that heat treatment of the lymphoid homogenate removes its ability to potentiate the lymphocytolytic activity of ACE. The spontaneous breakdown of washed lymphocytes in lymphoid homogenate is, however, not affected by heat treatment of lymphoid tissue factors.

Influence of Incubation Temperature: To determine the influence

TABLE 4. THE EFFECT OF HEAT-TREATED HOMOGENATES AND LOWERED INCUBATION TEMPERATURE UPON THE ACE LYMPHOCYTOLYTIC REACTION

Additions to cell suspension	Incubation temperature	Number of experiments	$\Delta\%$ ACE	S.B.*	
				Mean	Range
				<i>per cent</i>	<i>per cent</i>
ACE†+normal homogenate	38°	5	-10.6 ± 1.07	59	(35-81)
ACE +boiled homogenate‡	38°	5	$+2.0 \pm 2.35$	58	(30-75)
ACE +normal homogenate	38°	5	-11.0 ± 1.38	40	(25-65)
ACE +normal homogenate	5°	5	-0.8 ± 1.82	18	(6-25)

* S.B. Spontaneous breakdown.

† ACE, 10 $\mu\text{g. per cc.}$

‡ Homogenate placed in steam bath for 15 minutes, cooled and then rehomogenized.

of the incubation temperature upon the reaction, 10 μ g. per cc. ACE plus lymphoid homogenate was incubated with "non-washed" lymphocytes at both 38° and 5° C. for one hour. The results of five experiments, where in each case the same cells and homogenate were employed, are also illustrated in Table 4. It will be seen that the lymphocytolytic activity of ACE is significantly reduced when the reaction proceeds at 5° as compared to the usual incubation temperature of 38° C. It will also be noted that the spontaneous breakdown of lymphocytes is inhibited by the lower temperature.

Fractionation of Homogenates by Centrifugation: In the next experiments, lymphoid homogenates were fractionated by centrifugation and the activity of each fraction compared to that of the whole homogenate.

Upon centrifugation of lymphoid homogenates at approximately 2,000 RPM for 30 minutes, the homogenate yields three separate phases. The *bottom* of the centrifuge tube contains the insoluble particles; in the *middle*, there is a pink-colored cloudy, soluble fraction; and at the *top* there is a small white, fatty layer. The activity of these fractions relative to the whole homogenate was studied in 5 experiments. In each experiment "washed" lymphocytes from a single suspension were used; the lymphoid homogenate was prepared by homogenizing the tissue with 0.5 per cent sodium chloride containing M/30 phosphate buffer (pH 7.0). One portion of homogenate was set aside while the other was centrifuged. The bulk of the "top" fraction was removed with a spatula and resuspended in a volume of saline-phosphate equal to the original volume of the homogenate portion subjected to centrifugation. It was not possible to remove the "top" fraction quantitatively and thus the "middle" fraction was contaminated to some degree. The "middle" fraction was then removed with a pipette; and the "bottom" fraction was washed with saline, resuspended in saline-phosphate, and brought to its original volume. In addition to ACE, the effect of cholesterol (10 μ g. per cc.) was simultaneously studied.

The results of these fractionation studies are shown in Table 5 and indicate that the activity present in lymphoid homogenate tends to be concentrated mainly in the "top" fraction, although there is some activity in the "middle" fraction while the "bottom" fractions are inactive. In three experiments where the "top" fraction of lymphoid homogenates was heated for 15 minutes at 100° and then incubated with "washed" lymphocytes in the presence of ACE, lymphocytolytic activity was absent.

In summary the findings presented in this section suggest that there is present in lymphoid homogenates a heat-labile factor which significantly increases the lymphocytolytic activity of ACE. This factor is ineffective at low temperatures and appears to be associated with the "light" lipid particles in homogenates of lymphoid tissue.

TABLE 5. VARIOUS FRACTIONS OF LYMPHOID HOMOGENATE UPON THE ACE LYMPHOCYTOLITIC REACTION

Experi- ment†	Initial count ×10 ³	Whole homogenate			Top fraction			Middle fraction			Bottom fraction		
		S.B.*	Δ% ACE	Δ% CHOL.	Δ% ACE	Δ% CHOL.	Δ% ACE	Δ% CHOL.	Δ% ACE	Δ% CHOL.	S.B.*	Δ% ACE	Δ% CHOL.
1	cells per mm. ³	per cent			per cent		per cent				per cent		
2	30.2	70	-18	-5	-17	+2	-15	-10	-17	81	-17	-3	
3	28.5	46	-27	-4	-11	+5	-11	-3	-7	52	-7	-5	
4	43.0	48	-13	-2	-14	+2	-8	-1	+9	75	+9	+2	
5	92.5	78	-12	+8	-13	-4	-9	+1	-13	73	-13	-14	
	99.5	55	-14	-3	-17	-5	+9	-8	+7	46	+7	-3	
Mean		59	-16.8	-1.2	-14.5	0	-6.8	-4.2	-4.2	65	-4.2	-4.6	

† "Washed" lymphocytes used.

* Refers to the percentage lymphocyte breakdown in the absence of steroids, upon incubation for 1 hour at 38° C.

Effect of Crystalline Steroids

In considering the specificity of the ACE lymphocytolytic reaction, our attention was directed primarily to three questions. (a) Is the ACE effect described non-specific in the sense that any hormonal steroid would reproduce the effect of ACE, *i.e.*, activation by lymphoid homogenate? (b) Would other steroids possess lymphocytolytic activity in the absence of the lymphoid tissue factor? (c) Can the lymphocytolytic activity of ACE be accounted for on the basis of a single crystalline corticosteroid known to be present in the whole extract, for example, Compound E of Kendall?

TABLE 6. THE EFFECT OF VARIOUS STEROIDS UPON LYMPHOCYTES INCUBATED IN SERUM OR LYMPHOID HOMOGENATES FOR ONE HOUR AT 38° C.

Steroids*	"Non-washed" lymphocytes				"Damaged" lymphocytes	
	Lymphoid homogenate		Serum		Lymphoid homogenate	
	Number	Mean Δ%	Number	Mean Δ%	Number	Mean Δ%
ACE	22	<i>-16.8</i> ± 1.6†	13	- 5.3 ± 1.9	13	-12.0 ± 1.6
α-Estradiol	10	<i>-12.6</i> ± 1.8	15	<i>-11.5</i> ± 1.7	—	—
17-hydroxyprogesterone	6	- 8.3 ± 2.0	9	- 7.1 ± 2.0	—	—
Ethyl testosterone	6	- 2.2 ± 2.3	12	<i>-12.4</i> ± 2.8	—	—
Desoxycorticosterone acetate	10	- 2.1 ± 2.3	10	- 4.2 ± 3.8	6	- 0.8 ± 2.5
Desoxycorticosterone	4	+ 1.7 ± 0.8	—	—	6	- 2.7 ± 2.4
Corticosterone	10	- 1.3 ± 2.9	12	- 5.0 ± 2.6	6	+ 4.3 ± 3.1
Compound A	11	- 1.1 ± 3.4	14	- 5.5 ± 3.4	6	- 0.7 ± 2.1
Compound E	4	- 0.3 ± 2.1	—	—	6	+ 1.8 ± 2.6
Androsterone	6	+ 1.8 ± 3.6	6	- 0.2 ± 1.7	—	—
Cholesterol	8	+ 1.5 ± 1.5	12	- 1.2 ± 1.4	11	+ 1.1 ± 1.1

* Steroid concentration 10 μg. per cc.

† All values which are italicized are statistically significant ($p < 0.05$) as compared to the appropriate cholesterol control group.

‡ Standard error of the mean.

To obtain preliminary information on these points a number of crystalline steroids, both corticoid and non-corticoid were tested at a single dosage level of 10 μg. per cc. upon lymphocytes incubated for one hour at 38° C. Table 6 presents the collected data of experiments where various steroids were tested upon "non-washed" and damaged lymphocytes incubated in either serum or lymphoid homogenates. The data may be summarized briefly as follows:

(1) None of the steroids tested exhibit the characteristic response induced by ACE; *i.e.* inactivity in serum, activation by lymphoid homogenate.

(2) Of the crystalline cortical hormones tested, some of which are known to be present in ACE, none possess significant lymphocytolytic activity at a dosage level of 10 μg. per cc. in the presence of either lymphoid homogenate or serum.

(3) Of the four non-cortical hormonal steroids tested, only androsterone is without any significant lymphocytolytic activity. Estradiol is equally effective as a lymphocytolytic agent upon cells incubated either in homogenates or serum. 17-hydroxy progesterone, although somewhat less active, behaves similarly. Ethyl testosterone is significantly effective against lymphocytes incubated in serum, but in-

effective in the presence of homogenates, a situation which is the reverse of that found with ACE.

To rule out the possibility that the results with estradiol upon "non-washed" cells incubated in serum are due to the possible presence of the lymphoid tissue factor present as a contaminant in "non-washed" cell suspensions, a second series of eight experiments with estradiol using "washed" lymphocytes was performed. The results obtained were essentially similar to those using "non-washed" cells the mean $\Delta\%$ of estradiol being -12.9 ± 1.8 upon "washed" cells.

DISCUSSION

In accord with the working hypothesis upon which this study was initiated, it has been found that ACE is an effective lymphocytolytic agent in the presence of homogenates of lymphoid tissue, but that ACE is ineffective in serum, blood or buffered saline solutions. Preliminary studies suggest that the lymphoid tissue co-factor is enzymatic, since heat treatment of homogenates or decreasing the reaction temperature abolishes the ability of lymphoid homogenates to potentiate ACE lymphocytolytic activity. Fractionation studies of lymphoid homogenates by centrifugation indicate that the ACE co-factor is associated with the "light lipoid" fraction of homogenates; however, the question of whether this association is real or merely an artifact remains to be determined.

The lymphocytolytic activity obtained with ACE in the presence of lymphoid homogenates, while small, is highly significant statistically. Moreover, the lymphocytolytic activity of ACE was consistently observed in experiments using three different types of lymphocyte suspensions. Considering the results with the "washed" and "non-washed" lymphocytes, ACE produced an average $\Delta\%$ value of -15 , which means that 15 cells per 100 cells of the control were broken down by ACE during one hour of incubation. This value is equivalent to increasing the percentage lymphocyte breakdown per hour from an average value of 38 per cent in the absence of ACE, to 48 per cent in the presence of ACE. While these figures of ACE action appear small, a $\Delta\%$ value of -15 in a system containing 4.0 cc. total volume, where the control contains 100,000 cells per mm^3 , represents the breakdown of 6×10^7 lymphocytes by 40 μg . of ACE in one hour. When it is considered that the limiting factor in the reaction described may be the concentration of lymphoid co-factor and that lymphocytolysis was measured at a single arbitrary interval (which may not give maximal results) it is apparent that in future experiments greater *in vitro* effects of ACE may well be achieved.

Contrary to expectations, it was observed that homogenates of brain likewise potentiate ACE lymphocytolytic activity. It thus seems clear that the ACE co-factor hypothesized is not present exclusively in lymphoid tissue. The distribution of similar factors in other tissues awaits further experimentation. It should be empha-

sized, however, that the ACE accessory factor need not be distributed generally throughout the body tissues, since homogenates of muscle are completely inactive in potentiating ACE. The presence of an accessory factor for ACE lymphocytolytic activity *in vitro* in lymphoid tissue, a major site of lymphocyte breakdown *in situ*, would appear to have clear physiological significance for an understanding of ACE action upon lymphocytes. On the other hand, the presence of an ACE co-factor in brain is, as yet, completely inexplicable. The nature of the co-factor in brain, its relationship to the lymphoid tissue factor and its physiologic importance remains to be determined.

At the present time, nothing is known of the manner of action of the co-factors described. It would appear that they could act either by increasing the sensitivity of lymphocytes to ACE or contrariwise, by transforming ACE from an inactive to a potent lymphocytolytic agent. The available data does not permit differentiation between these basically dissimilar mechanisms. However, the finding that some steroids, such as estradiol, exhibit significant lymphocytolytic activity in the absence of lymphoid homogenates, may perhaps be taken as suggestive evidence favoring the view that the co-factors (enzymes?) transform one or more of the steroids in ACE to another compound which is the active agent. The idea that lymphoid homogenates act by transforming steroids rather than by altering the sensitivity of lymphocytes could likewise account for the finding that ethyl-testosterone, active against lymphocytes in serum, is inactive as a lymphocytolytic agent in the presence of lymphoid homogenates.

When steroids such as ACE or estradiol accelerate the rate of breakdown of lymphocytes incubated in lymphoid homogenate, they are producing this result upon cells whose cytoplasmic membrane is no longer functional, as evidenced by the fact that such cells are freely permeable to eosin. It thus seems unlikely that lymphocytolytic steroids act by altering the cytoplasmic membrane. The lymphocyte is largely nuclear, and lymphocytolysis as measured by our method in reality measures dissolution of nuclei, composed largely of desoxyribonucleoprotein complex (Mirsky and Pollister, 1946). It thus seems legitimate to consider the possibility that steroid hormone action upon lymphocytes involves an effect upon desoxyribonucleoproteins possibly via desoxyribonuclease or through other means.

In considering the steroid specificity aspects of the ACE lymphocytolytic reaction, it is apparent that our preliminary studies of crystalline steroids are incomplete since only a single dosage level was employed under arbitrarily selected conditions which had proved satisfactory for the demonstration of ACE activity. If other dosage levels had been tested in a manner designed to test activity in a kinetic fashion, it is possible that the results might be qualitatively different from those reported here. It is therefore apparent that it is not profitable to discuss these incomplete *in vitro* findings in relation to the known *in vivo* actions of the "carbohydrate-active" cortico-

steroids upon lymphoid tissue. Nevertheless, certain aspects of the steroid specificity studies are worthy of mention. Of the ten crystalline steroids tested not a single compound reproduced the results obtained with ACE (i.e. *inactivity* in serum, *activity* in the presence of lymphoid homogenate). To this extent, the results obtained demonstrate a degree of specificity for the *in vitro* lymphocytolytic reaction of ACE described. It is also of some interest that estradiol, ethyl testosterone and 17-hydroxyprogesterone all exhibit significant lymphocytolytic activity in the absence of the lymphoid cofactor, suggesting that these steroids act directly upon lymphocytes. While these non-corticoids can be differentiated from ACE in that they are not activated by lymphoid homogenates (ethyl testosterone is presumably inactivated by a factor present in lymphoid homogenates) it seems striking that these steroids should act upon lymphocytes *in vitro*, while corticosteroids like corticosterone, Compound A and Compound E are inactive. Is it possible that the *in vivo* specificity of hormone action upon lymphoid tissue is determined not so much by the specific configuration of the steroid molecule, but by factors (e.g. capillary and cell permeability) which determine the entry of steroids into the target organ in question?

The negative findings with crystalline corticosteroids tested indicate that the action of the ACE preparation tested cannot be explained on the basis of a single corticosteroid of the types employed. This suggests that ACE may contain a highly potent, as yet unidentified substance which is quantitatively dissimilar from the types of corticosteroid tested, or it may be that the lymphocytolytic activity of ACE is the result of synergistic action of two or more of the crystalline hormones tested?

It should not be inferred from these studies that lymphocytolytic activity is the only, or indeed the most important, action of adrenocortical steroids upon lymphocytes. The suggestion of Robertson (1948) that ACH controls the maturation and mitotic rate of young lymphocytes, the histological observation that ACH action *in vivo* may produce cytoplasmic dissolution of lymphocytes leaving the nucleus intact (White and Dougherty, 1946); and the findings of Schrek (personal communication) that ACE (in the absence of added lymphoid homogenate) increases lymphocyte permeability to eosin, without promoting cell lysis, may be taken to indicate that ACH affects not one but several factors involved in maintenance of the structural integrity of the lymphocyte.

One aspect of our results, although not directly related to steroid action upon lymphocytes, merits special attention. It should be noted that in the absence of added steroids, lymphoid homogenates exhibit marked lymphocytotoxic activity as evidenced by alteration of permeability, shrinkage and accelerated dissolution of lymphocytes. The toxic activity responsible for these effects appears to be a separate factor from that which potentiates ACE lymphocytolytic action.

Preliminary studies indicate it to be thermostable and absent in muscle and brain homogenates. The finding of a highly active lymphocytotoxic factor in lymphoid tissue appears to warrant further study.

SUMMARY

Under conditions where spontaneous lymphocytolysis is proceeding, it has been observed that ACE (10 μ g. per cc.) in the presence of lymphoid tissue homogenates significantly increases the rate of lymphocyte breakdown. ACE is likewise active with brain homogenates but inactive in the presence of homologous serum, or defibrinated blood, buffered salt solutions, and muscle homogenates. The factor present in lymphoid tissue homogenates may be an enzyme and appears to be associated with the light-lipoid particles of the homogenate. The lymphocytolytic reaction obtained with ACE seems to be specific in the sense that none of the ten crystalline steroids tested duplicate the results obtained with ACE. Estradiol, 17-hydroxy progesterone and ethyl testosterone all exhibit lymphocytolytic activity upon cells incubated in serum. None of these steroids, however, exhibit an increased lymphocytolytic activity with lymphoid homogenates and one of these steroids (ethyl testosterone) is inactive under these conditions. In the dosages tested, 11-dehydrocorticosterone, 17-hydroxy 11-dehydro corticosterone, corticosterone, 11-desoxycorticosterone (or its acetate) and androsterone do not exhibit lymphocytolytic activity either in the absence or presence of lymphoid homogenates. The limitations and possible significance of these *in vitro* findings are briefly discussed.

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THE EFFECT OF PREGNENOLONE ON REINITIATION AND MAINTENANCE OF SPERMATOGENESIS IN HYPOPHYSECTOMIZED RATS¹

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Δ^5 -PREGNENOLONE has recently been isolated from hog testicular extract (Haines, Johnson, Goodwin and Kuizenga, 1948). This steroid can maintain spermatogenesis in hypophysectomized rats (Leatham and Brent, 1943) and in estrogen treated rats (Albert and Selye, 1942). However, it is unable to reinitiate spermatogenesis in the atrophic testis following hypophysectomy or estrogen therapy (Masson, 1946). It seemed of interest to determine whether larger dosages or longer treatment periods would reinitiate spermatogenesis in hypophysectomized rats. The effect of pregnenolone on the maintenance of spermatogenesis also was restudied, using small dosages of hormone. Subcutaneous versus oral routes of administration were compared. The effect of small dosages of pregnenolone on testis and accessory sex glands of normal rats was also studied.

METHODS

Adult male rats of the Long-Evans strain were hypophysectomized and 20 days later received daily subcutaneous injections for 20 days of either 3 mg. or 20 mg. of pregnenolone or 4 mg. of pregnenolone acetate or 3 mg. of testosterone propionate.³ The left testis and epididymis were removed at the beginning of treatment and served as controls. In the maintenance experiments treatment with daily subcutaneous injections of 1 mg. of pregnenolone or 2 mg. of pregnenolone acetate or daily force feedings with 10 mg. of pregnenolone in aqueous suspension was started at the time of operation and continued for 20 days. Normal adult male rats were injected subcutaneously each day for 20 days with 0.1 mg. or 0.5 mg. or 2.5 mg. of pregnenolone.

At autopsy, the testes and sex accessory glands were weighed to the nearest milligram and fixed in Bouin's fluid. Sections were made at 8 μ and stained with hematoxylin and eosin. The pituitary capsules were serially sectioned, and only completely hypophysectomized animals are reported.

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RESULTS

Neither pregnenolone, in dosage up to 20 mg. per day, nor pregnenolone acetate were able to reinitiate spermatogenesis nor prevent the progressive decrease in testis or sex accessory gland weight during the period of 20 to 40 days following hypophysectomy (table 1). Testosterone propionate, under similar conditions, increased testis and sex accessory gland weight (table 1), and served to increase the number of cell layers and the diameter of the tubules of the testis. Spermatozoa did not form during the 20 day treatment period, however.

As little as 1 mg. of pregnenolone or 2 mg. of pregnenolone acetate subcutaneously per day maintained spermatogenesis for 20 days fol-

TABLE 1. WEIGHTS (MG.) OF TESTES, EPIDIDYMIDES, SEMINAL VESICLES AND VENTRAL PROSTATE OF ADULT RATS TREATED WITH THE INDICATED MATERIALS BY DAILY SUBCUTANEOUS INJECTION FOR 20 DAYS STARTING 20 DAYS AFTER HYPOPHYSECTOMY. AT THE START OF TREATMENT THE LEFT TESTIS AND EPIDIDYMS WERE REMOVED AND SERVED AS CONTROLS

Treatment	No. of animals	Testis Wt. Avg. and range		Epididymis Wt. Avg. and range		Seminal vesicles Avg. and range	Ventral prostate Avg. and range
		Before Tr.	After Tr.	Before Tr.	After Tr.		
Sesame Oil 0.75 cc./d.	1	333	155	109	84	52	15
Pregnenolone 3 mg./d.	2	343 (301-384)	164 (142-185)	88 (74-101)	80 (75-84)	55 (45-64)	9 (6-11)
Pregnenolone 20 mg./d.	2	249 (238-260)	164 (162-185)	76 (74-77)	63 (61-64)	35 (34-36)	8 (6-10)
Pregnenolone Acetate 4 mg./d.	3	299 (277-334)	134 (125-148)	75 (75-76)	65 (62-70)	57 (40-70)	10 (8-13)
Testosterone Propionate 3 mg./d.	2	239 (190-288)	309 (247-370)	84 (68-95)	198 (195-200)	299 (290-307)	229 (205-252)

lowing hypophysectomy and partially maintained testis weight (table 2). Seminal vesicle, prostate and adrenal weights were not maintained and resembled those of hypophysectomized controls. Epididymis weight was partially maintained (table 2). Oral administration of 10 mg. of pregnenolone per day was completely ineffective in maintaining spermatogenesis, and testis weight was similar to that of controls (table 2).

The administration of 0.1 mg. or 0.5 mg. or 2.5 mg. of pregnenolone per day for 20 days to normal rats failed to significantly alter testis, sex accessory gland or adrenal or pituitary gland weight. Testis structure remained completely normal.

DISCUSSION

The presence of a steroid in testis tissue which is effective in maintaining spermatogenesis and which is completely devoid of androgenic ability is of considerable theoretical importance. It raises

TABLE 2. WEIGHTS (MG.) OF TESTES, SEX ACCESSORY GLANDS AND ADRENALS OF ADULT HYPOPHYSECTOMIZED RATS TREATED DAILY FOR 20 DAYS FOLLOWING OPERATION WITH SUBCUTANEOUS INJECTIONS OF 1 MG. OF PREGNENOLONE IN 0.25 CC. SESAME OIL OR 2 MG. OF PREGNENOLONE ACETATE IN 0.25 CC. OF SESAME OIL, OR 0.25 CC. SESAME OIL ALONE, OR FORCED FED WITH 10 MG. OF PREGNENOLONE IN 2.0 CC. OF WATER

Treatment	No. of animals	Testes		Epididymides		Seminal vesicles		Ventral prostate		Adrenals	
		Av.— Range	Contr. =1.00	Av.— Range	Contr. =1.00	Av.— Range	Contr. =1.00	Av.— Range	Contr. =1.00	Av.— Range	Contr. =1.00
Pregnenolone 1 mg./d. subcutaneously	3	1734 (1368-2383)	2.12	403 (304-550)	1.56	87 (66-98)	1.00	25 (15-32)	1.14	13 (12-14)	1.00
Pregnenolone Acetate 2 mg./d. subcutaneously	1	1240	1.52	351	1.35	80	0.92	22	1.00	11	0.85
Oil Injected Controls	2	818 (700-936)	1.00	259 (224-295)	1.00	87 (75-98)	1.00	22 (21-23)	1.00	13 (13-14)	1.00
Pregnenolone 10 mg./d. orally	4	777 (695-868)	0.95	236 (195-263)	0.91	84 (65-93)	0.97	26 (24-28)	1.09	14 (14-15)	1.08

the possibility that pituitary F.S.H. action on the tubules may be mediated by the release of such a steroid which would have no influence on the accessory sex glands.

Though most steroids which are able to maintain spermatogenesis in hypophysectomized rats are also androgens, there is no definite correlation between these actions (Nelson, 1937). Even weakly androgenic progesterone has spermatogenesis maintaining ability (Nelson, 1938; Seyle and Friedman, 1941).

The absence of any inhibitory effect of pregnenolone on pituitary gonadotrophin secretion, as judged by the lack of effect of small dosages on normal testis and accessory gland weight and structure, is also of considerable interest.

The ability of pregnenolone to maintain spermatogenesis contrasts with its inability to reinitiate spermatogenesis. This confirms Masson's (1946) findings, and is similar to the early reports with testosterone (Cutuly, McCullagh and Cutuly, 1938; Hamilton and Leonard, 1938). Later experiments, using larger dosages of testosterone for longer periods (Nelson, 1940, 1941, 1946), or small dosages administered by intratesticular pellet implant (Dvoskin, 1947), demonstrated testosterone to be effective in reinitiating spermatogenesis in hypophysectomized rats. In the present experiments, testosterone failed to reinitiate spermatogenesis in a 20 day period, though it definitely increased testis weight and caused an increase in the size and number of cells layers in the tubules. This did not occur in the pregnenolone treated rats, and may remotely be due to the short (20 day) treatment period, though some effect should be expected in this time.

Pregnenolone has also recently been found to be without effect on sperm count in human infertility (Tyler, Payne and Kirsch, 1948).

CONCLUSIONS

Neither pregnenolone in dosages of 3 mg. or 20 mg. per day or pregnenolone acetate in dosages of 4 mg. per day were able to reinitiate spermatogenesis or prevent progressive testis weight loss during a 20 to 40 day period after hypophysectomy.

Small dosages of pregnenolone (1 mg. per day) or pregnenolone acetate (2 mg. per day) subcutaneously administered were able to maintain spermatogenesis and partially maintain testis weight for 20 days after hypophysectomy. Sex accessory gland weight, except for epididymis, were similar to untreated hypophysectomized controls.

Orally administered pregnenolone in dosage of 10 mg. per day was completely ineffective in maintaining spermatogenesis in hypophysectomized rats.

Pregnenolone in dosage of 0.1 mg. or 0.5 mg. or 2.5 mg. per day had no effect on testis or sex accessory gland weight in normal rats.

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THE ANTIGOITROGENIC ACTION OF ELEMENTAL IODINE

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Dvoskin (1947 a and b) has presented evidence that elemental iodine injected into rats can be converted into thyroxin or thyroxin-like material in tissues other than the thyroid gland. The observations presented here give some support to this hypothesis, but our experimental results differ so greatly from those of Dvoskin that they seem worth reporting.

In Dvoskin's experiments, the injection of elemental iodine prevented entirely the goitrogenic action of thiouracil given orally or by implantation. In our experiments elemental iodine given by injection had only a limited antigoitrogenic action which was no greater than that of iodides given orally. Evidence of extra-thyroidal formation of thyroxin-like material from elemental iodine was apparent only in experiments on metabolic rate.

METHODS

White male rats of a Wistar strain, weighing between 135 and 175 gm. were given thiouracil 0.1% in drinking water. Eight rats were used in each group (Table 1). One control group received thiouracil only. Another control group received (in addition to thiouracil) daily subcutaneous injections of mineral oil. Five other groups received daily subcutaneous injections of various amounts of elemental iodine in mineral oil. The animals were killed after 14 days and the thyroid glands were removed and weighed. In some experiments propylene-glycol was used as the solvent for iodine and one experiment was performed with iodine trichloride.

The experiments on oxygen consumption were done by a closed circuit technique, using a battery of eight chambers in a water-bath at 30°C., with one rat in each chamber. All the animals received thiouracil (0.1% in drinking water). Four of each group of eight were injected daily with iodine in mineral oil (1 mg. in 0.2 cc.). Measurements were made at the same time each morning, six days a week, on all eight rats simultaneously, without preliminary fasting. The figure used for the initial metabolic rate was the average of five measurements made before treatment was started. The results were calculated as cc. of oxygen per sq. meter of body surface per minute. The final figure was the average of the last five days of fifteen days of treatment. During the last five days of this period the rate of change of metabolic rate

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was perceptible on inspecting the figures, but small enough to justify averaging them. The results are presented in Table 2 as per cent decrease in oxygen consumption. The per cent difference for each animal was treated as a single figure and the S.E. shown in Table 2 indicates the variability among the four animals in each group. The rats used weighed between 125 and 180 gm.

OBSERVATIONS

It is apparent that in our experiments elemental iodine by injection did not prevent the goitrogenic action of thiouracil. The diminution in enlargement which occurred was only of the order of magnitude

TABLE 1. ANTIGOITROGENIC ACTION OF ELEMENTAL IODINE BY INJECTION.
DURATION TREATMENT 2 WEEKS

No.	Experimental groups	Thyroid wt. mg./100 gm. \pm S.E
1	Controls (Thiouracil only, no injections)	26.5 \pm 1.5
2	Controls (Thiouracil + 0.2 cc. oil injected)	27.8 \pm 1.6
3	I ₂ 5 mg./d. in 0.2 cc. of oil	22.7 \pm 1.6
4	I ₂ mg./d. in 0.2 cc. of oil	24.5 \pm 0.9
5	I ₂ 1 mg./d. in 0.2 cc. of oil	25.3 \pm 1.6
6	I ₂ 0.5 mg./d. in 0.2 cc. of oil	24.2 \pm 1.5
7	I ₂ 0.1 mg./d. in 0.2 cc. of oil	22.7 \pm 1.4
8	I ₂ 5 mg./d. in 0.2 cc. of propylene glycol	23.4 \pm 1.4
9	I ₂ 1 mg./d. in 0.2 cc. of propylene glycol	24.2 \pm 1.6
10	ICl 1 mg./d. in 0.2 cc. of propylene glycol	20.9 \pm 0.7
11	Untreated normals	10.2 \pm 0.4

which we have reported elsewhere as resulting from iodides given orally (Ferguson and Sellers 1948) and which has been often observed by other investigators.

The thyroid glands from the animals used in the experiments on oxygen consumption were also excised and weighed, and showed practically the same degree of partial antigoitrogenic action by injected iodine as shown in Table 1.

TABLE 2. EFFECT OF ELEMENTAL IODINE AND THIOURACIL ON OXYGEN CONSUMPTION.
FOUR RATS PER GROUP; 1.0 MG. OF IODINE DAILY FOR 15 DAYS

Expt. No.	Average per cent decrease in O ₂ consumption \pm S.E.	
	Thiouracil only	Thiouracil plus iodine
1	15.0 \pm 1.8	-0.7 \pm 1.7
2	30.0 \pm 1.7	18.5 \pm 3.1
3	15.6 \pm 3.5	5.4 \pm 2.9

From Table 2, however, it appears that elemental iodine does materially alter the metabolic response to thiouracil, as though the injected iodine had been partly converted to thyroxine. The injected iodine in one case maintained, and in the other cases partially sustained the rate of oxygen consumption. This result differs from the effect of iodides given orally which did not affect the decline of oxygen

consumption resulting from the administration of thiouracil (Ferguson and Sellers 1948). Although none of our animals showed ulceration or excessive induration at the sites of injection, the possibility that local irritation might increase the metabolic rate and thus explain our results could not be disregarded. However, when four rats which had been receiving propylthiouracil for several months were injected subcutaneously with 0.2 cc. of 5% aqueous formalin, producing a comparable indurated lesion, the metabolic rates actually decreased slightly.

DISCUSSION

Although our observations are consistent with Dvoskin's hypothesis, the thyroxin-like action of injected iodine was much weaker than in Dvoskin's experiments, and evident only in experiments on metabolic rate. Metabolic experiments were not reported by Dvoskin. Two possible explanations suggest themselves. (1) Our rats were all older and larger than those used by Dvoskin. It is possible that the capacity for producing thyroxin extra-thyroidally decreases with age. (2) It is possible that genetic differences in our animals may be responsible for our differing results.

Since these experiments were done, Barker and Lipner (1948) have reported that the protein bound iodine and the metabolic rate of thyroidectomized rats and rats treated with thiouracil increase when elemental iodine is injected. There is thus considerable evidence that thyroxin-like material may be formed outside the thyroid gland.

SUMMARY

Elemental iodine by injection did not prevent the enlargement of the thyroid gland produced in white rats by thiouracil given orally.

A limited antigoutrogenic action resulted from elemental iodine by injection but the magnitude was no greater than that often observed with iodides given orally.

Elemental iodine by injection lessened the rate of decrease of metabolic rate under treatment with thiouracil, while iodides had no such action. This observation is consistent with the hypothesis that some thyroxin-like material can be produced from elemental iodine in extra-thyroid tissues.

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INACTIVATION OF POSTERIOR PITUITARY ANTI-DIURETIC HORMONE BY THE LIVER¹

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IT HAS long been recognized that the liver bears some important relation to water metabolism (reviewed by Pick, 1929 and 1946; Ratnoff and Patek, 1942). In experimental liver damage (Adlersberg, 1934; Adlersberg and Fox, 1943) and in liver disease of man (Labby and Hoagland, 1947; Ralli *et al.*, 1945; Leslie *et al.*, 1948) water retention has been reported. The presence of antidiuretic substances in urine has been associated with liver dysfunction (Ralli *et al.*, 1945; Leslie and Ralli, 1947; Hall, Frame and Drill, 1949). The suggestion has been made that this may be the well known neurohypophyseal antidiuretic hormone (ADH) appearing in the urine because it is not effectively inactivated by the damaged liver (Ralli *et al.* 1945).

ADH is inactivated at some body site as judged by the small amounts excreted after injection (Heller and Urban, 1935; Ingram, Ladd and Benlbow, 1939) and the *in vitro* studies of Heller and Urban (1935) indicate that while kidney and blood inactivate ADH, liver tissue is more effective.

To test the role of the liver in the inactivation of posterior pituitary antidiuretic hormone, we have studied the response of animals to Pitressin administered through an hepatic portal drainage as compared with the response of those which received the hormone by other routes. This technique has been effectively employed in studying the role played by the hepatic tissue in the inactivation of other hormones. In addition, the antidiuretic activity of Pitressin was tested after incubation with extracts of several different tissues.

Some of these observations were recorded in an earlier abstract (Eversole, Birnie and Gaunt, 1948).

MATERIAL AND METHODS

Mature male rats weighing approximately 200 grams were used in the water excretion tests. They were fasted but allowed water for 18 hours prior to testing. At the end of the fast, one dose of distilled water amounting to 4 cc. per 100 sq. cm. of body surface was warmed to 100°F and administered

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by stomach tube. The dose for a 200 gram rat was 12.5 cc. with body surface calculated according to the formula of Benedict (1938). The animals were placed in individual metabolism cages and the urine output measured at 30 minute intervals for a period of 3 hours. Since there is a day to day variation in the diuretic response to water, equal numbers of controls were run simultaneously with experimental animals.

All animals were anesthetized with ether and in those receiving intrasplenic injections, the spleen was withdrawn from the body cavity through a subcostal incision. In the experimental rats, injections of 0.1 cc. of freshly-diluted Pitressin solution was made directly into the exposed spleen with a 27 gauge hypodermic needle three-quarters of an inch long. In addition, 0.1 cc. of water was given subcutaneously because control animals received Pitressin under the skin. Immediately after injection, the spleen was returned to the body cavity and the incision sutured. Control animals were given Pitressin subcutaneously or intramuscularly in amounts equal to that given the experimental cases. In addition, the spleen of controls was exposed and manipulated as above and injected with 0.1 cc. of water.

For intrahepatic injections the animals were anesthetized with ether, and the left lateral lobe of the liver forced gently through a midventral incision. Pitressin injections were made into the exposed liver lobe, the incision sutured and 0.1 cc. of water was given subcutaneously. Control animals were injected subcutaneously with Pitressin and with water intrahepatically.

Pitressin was given 30 minutes before or 30 minutes after the oral administration of the test dose of water. Hormone and water could not be given satisfactorily at the same time because the animals had to be anesthetized for the hepatic and splenic injections and water administered to etherized rats backs up from the stomach, preventing control of dosage.

For the *in vitro* studies, extracts were prepared from tissues immediately after their removal from rats killed by a blow on the head. The extracts were prepared by grinding 1 gram of tissue in a mortar with sand and extracting with 10 cc. of 1 per cent saline for 10 minutes. The homogenates were centrifuged at 2,600 R.P.M. for 10 minutes and the supernatant fluids tested for their ability to inactivate Pitressin. The extracts were adjusted to pH 6.8, the desired amount of Pitressin added, and incubated for 20 minutes at 37°C. Following incubation, 1 cc. of this material was injected intraperitoneally into hydrated animals and tested by the method of Birnie *et al.*, (1949) to determine the amount of antidiuretic material present.

RESULTS

The injection of 40 milliunits of Pitressin into the spleen was less effective in suppressing diuresis than equal doses administered by subcutaneous injection. The findings summarized in series 1 of Table 1 are from animals receiving Pitressin 30 minutes before the water test. The differences were apparent throughout the 3 hours of observation and were shown to be statistically significant at the 90 minute interval ($P=.02$).

In the groups shown in series 2 and 3 of Table 1, the Pitressin was injected 30 minutes after water administration. Under these circumstances the antidiuretic effects of posterior pituitary extract were

more pronounced by all routes of administration than when given before the water. In series 2, intramuscular injections were markedly more effective than those given in the spleen. In series 3 in which the hormone was injected directly into the liver, its antidiuretic effects seemed to be reduced as compared with controls but no significant difference was established.

TABLE 1. ANTIDIURETIC RESPONSE TO 40 MILLIUNITS OF PITRESSIN GIVEN BY DIFFERENT ROUTES

Series	Route of administration of pitressin	No. rats	Percentage water excreted at indicated minutes					
			30	60	90	120	150	180
Pitressin given 30 minutes prior to water								
1	Intrasplenic	21	0.4	12.0	41.8	72.4	88.6	94.9
	Subcutaneously	18	0.0	5.6	29.1	63.7	81.6	87.4
Pitressin given 30 minutes after water								
2	Intrasplenic	6	5.9	6.4	18.3	41.8	70.0	83.1
	Intramuscular	6	0.7	0.7	8.2	28.1	50.4	67.9
3	Intrahepatic	5	0.0	3.3	25.1	49.2	73.4	81.5
	Subcutaneously	5	0.0	2.4	15.1	41.8	65.8	79.7

Smaller doses of Pitressin (10, 5, and 2 milliunits) were also used but variability was great and significant differences between subcutaneous and intrasplenic administration were not demonstrated. A more sensitive testing procedure might well reveal true differences although it is also possible that an exhibition of the effect is dependent upon a fairly high concentration of the hormone in body fluids.

TABLE 2. DISTRIBUTION OF PITRESSIN-INACTIVATING SYSTEM IN RAT TISSUE EXTRACTS (20 MILLIUNITS OF PITRESSIN ADDED TO EACH CC. OF TISSUE EXTRACT)

Tissue	No. cases	Percentage water excreted at 60 minutes
Liver	15	58.0
Kidney	6	22.6
Whole blood	6	11.3
Skeletal muscle	6	8.4
Heated liver extract*	6	1.7

* Heated to 80° C. for 5 minutes.

In vitro studies revealed that systems capable of inactivating Pitressin were present in cell-free extracts of rat liver, kidney and blood. The greatest activity per gram of tissue was found in liver extracts (Table 2). In this table a high percentage of water excretion is indicative of Pitressin inactivation while active Pitressin causes an inhibition of diuresis.

DISCUSSION

The experiments reported here show that when 40 milliunits of Pitressin is injected into a site with hepatic portal drainage, it is less effective than when introduced into the general circulation. Such results are most easily interpreted by the theory that the liver has an important role in the inactivation of circulating posterior pituitary antidiuretic hormone. The previous finding of Heller and Urban (1935) that homogenates of liver tissue rapidly inactivate posterior pituitary extract and the demonstration here that cell-free extracts of liver have the same action, provides further evidence for this view. This does not mean that the liver is the sole site of inactivation of the antidiuretic hormone. Our *in vitro* work and that of Heller and Urban indicate that other organs inactivate Pitressin but that they are less effective than the liver.

The action of Pitressin introduced directly into the liver differed less from controls run simultaneously than did that injected into the spleen. This is perhaps due to the fact that absorption of the material could be directly into efferent vascular channels of the liver. In that case it might be exposed less to the action of liver tissue.

We have no satisfactory explanation for the failure to demonstrate differences in effectiveness of small doses of Pitressin (10, 5 and 2 milliunits) when given by intrasplenic as compared with other injection routes. There is the possibility that the test methods used were sensitive enough to detect only those effects resulting from high initial blood levels of the material.

Our results would be consistent with the hypothesis (Labby and Hoagland, 1947; Ralli *et al.*, 1945) that the water retention seen in human liver disease is due to a decreased rate of inactivation of the antidiuretic hormone by damaged liver tissues. We could not explore this idea experimentally because of inability to produce in rats a liver damage characterized by water retention. Until such experiments can be done, no definite conclusions are warranted concerning the relation of our findings to the effects of liver disease. The diseased liver itself may be producing antidiuretic materials (Shorr and Zweifach, 1948).

SUMMARY

When 40 milliunits of Pitressin were injected into the spleen of hydrated rats the antidiuretic effect was less than when the injections were made subcutaneously or intramuscularly. Such differences were not seen at lower dose levels.

Cell-free extracts of liver tissue inactivated Pitressin more effectively than extracts of kidney or whole blood.

These results indicate that the liver is one site of inactivation of the posterior pituitary antidiuretic hormone.

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THE EFFECT OF TOTAL BODY X-RADIATION ON 17 KETOSTEROID EXCRETION IN DOGS¹

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ATTENTION HAS been drawn to alterations in adrenal cortical function in response to x-radiation (Narat, 1922; Jenkenson and Brown, 1944; Desjardins, 1928; Weichert, 1942; Hirsch, 1922; Selye, 1946). Histological and chemical alterations in the cortex in response to X-ray have been noted (Englestad and Torgerson, 1939; Weichert, 1942; Ellinger, 1948). Metabolic studies on patients exposed to X-ray have shown electrolyte changes which may be related to altered adrenal function (Goldman, 1943; Robertson, 1943). Several investigators have noted the absence of the indirect lymphopenic response to X-radiation in adrenalectomized animals (Leblond and Segal, 1942; Halberstaedler and Ichowicz, 1947; Dougherty and White, 1946). Lead shielding of the adrenal has been reported as having a positive effect on reducing the mortality to X-ray in a small number of male rats (Craver, 1946). Patt, *et al.*, (1947) have shown changes in the adrenal cholesterol content and adrenal weight following LD₅₀ and higher doses of radiation. More recently they have shown this response can be prevented by hypophysectomy (Patt, 1948). Cortical replacement therapy of radiation sickness has been reported (Ellinger, 1947; Ellinger, 1948; Ellinger, *et al.*, 1949; Weichert 1942).

As the foregoing studies were designed to be carried out on patients with disease, adrenalectomized animals, and animals sacrificed during the experiment, it seemed desirable to undertake a study where the intact animal served as his own control and where hormone excretion studies were followed directly throughout the post-radiation period. Seventeen ketosteroid analyses were best adapted to such a study. Seventeen ketosteroids have been shown to rise for a short period and then fall with a final reversion of excretion to normal levels in response to trauma and disease (Forbes, *et al.*, 1947; Stevenson, Shenker and Browne, 1944; Cope, Nathanson, Rourke and Wilson, 1943). This hormonal excretion pattern is observed in the adaptation of the organism to stress as described by Selye (1946). The sources of this steroid are both the testes and the cortex in the male and the cortex alone in the female (Fraser, Albright, *et al.*, 1941).

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¹ The opinions are those of the author and are not to be construed as reflecting the views of the Navy Department.

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Therefore, this test would give an indication of at least one aspect of adrenal cortical function.

METHODS

Eleven dogs, eight males and three females, weighing from 9 to 19 kg. were used in the course of the experiment. These dogs were maintained on Purina dog chow and water *ad libitum*.

The dogs were irradiated in plywood cages, 26 cm. wide, which had been constructed so that the maximum intensity of the angular beam of the X-ray was located at the horizontal mid-point of the dog. The cages were constructed to conform to a segment the arc of which was one meter (Chapman,

TABLE 1. NORMAL 17 KETOSTEROID EXCRETION VALUES IN MGM/24 HOURS

Dog	Sex	Wt./kg.	No. of determi- nations	Mean	Standard devia- tion	Range
49	F	9.3	12	1.05	0.59	0.23-2.00
47	F	12.4	13	1.65	1.41	0.39-4.86
37	F	11	11	0.85	0.58	0.23-2.40
21	M	12	10	1.35	0.65	0.73-2.63
32	M	12.2	13	1.65	1.28	0.51-5.14
50	M	10.4	10	0.75	0.38	0.22-1.34
27	M	16	30	1.82	0.79	0.42-2.95
23	M	16	20	1.85	1.09	1.75-5.43
6	M	17	22	3.06	0.63	0.65-5.19
1	M	14	12	2.04	1.30	0.77-3.44
40	M	19	39	1.69	0.86	0.43-4.89

et al., 1948). The radiation factors were: 1,000 KV, 3 ma, with an average intensity of 25.5 r/minute. The depth dosage using a pressedwood phantom of uniform density was 61 percent at 10 cm. and 13 percent at 25 cm. Dogs were radiated in two groups. Group one was composed of five male mongrels. Group two was composed of three male and three female purebred beagles as it was felt that this would render the biological response more uniform. A dose of 150 r was delivered to one side; the animals were turned around and 150 r was delivered to the other side for a total of 300 r. This had been determined as a probable minimal lethal dose at another laboratory (Boche, 1948).

Urine samples were collected in metabolism cages on a 24-hour basis. The washed neutral ether extract was then assayed for 17 ketosteroid content by the Zimmerman reaction as modified by Callow (1938) and Talbot (1940). Anhydrous aldehyde-free alcohol, purified 2 per cent m-dinitrobenzene, and 2.5 N alcoholic potassium hydroxide were used. The reaction was developed for one hour at $25^{\circ}\text{C} \pm 0.2^{\circ}\text{C}$ in darkness. The optical density of the colorimetric reaction was measured in an Evelyn photoelectric colorimeter using a filter having a maximum transmission at 520 m μ . The colorimeter was calibrated with known concentrations of crystalline dehydroisoandrosterone.

The first group of dogs was subjected to the minor stresses of being bled small amounts and of being transported in irradiation cages to the X-ray tube. They also received a dosage of 15 mgm. and 25 mgm. of purified adrenocorticotrophic hormone subcutaneously in an attempt to study adrenal reserve. These dosages were administered on different days three weeks before and ten days following radiation.

RESULTS

The normal values are recorded in table 1 as the number of determinations, the range, and the standard deviation of pre-irradiation values. These values agree with those reported elsewhere in the literature (Pashkis, Cantarow, *et al.*, 1943). These authors report no sexual difference. In the five dogs comprising the first group, minor stresses and a single dosage of adrenocorticotrophic hormone produced no significant change in the 24-hour 17 ketosteroid excretion.

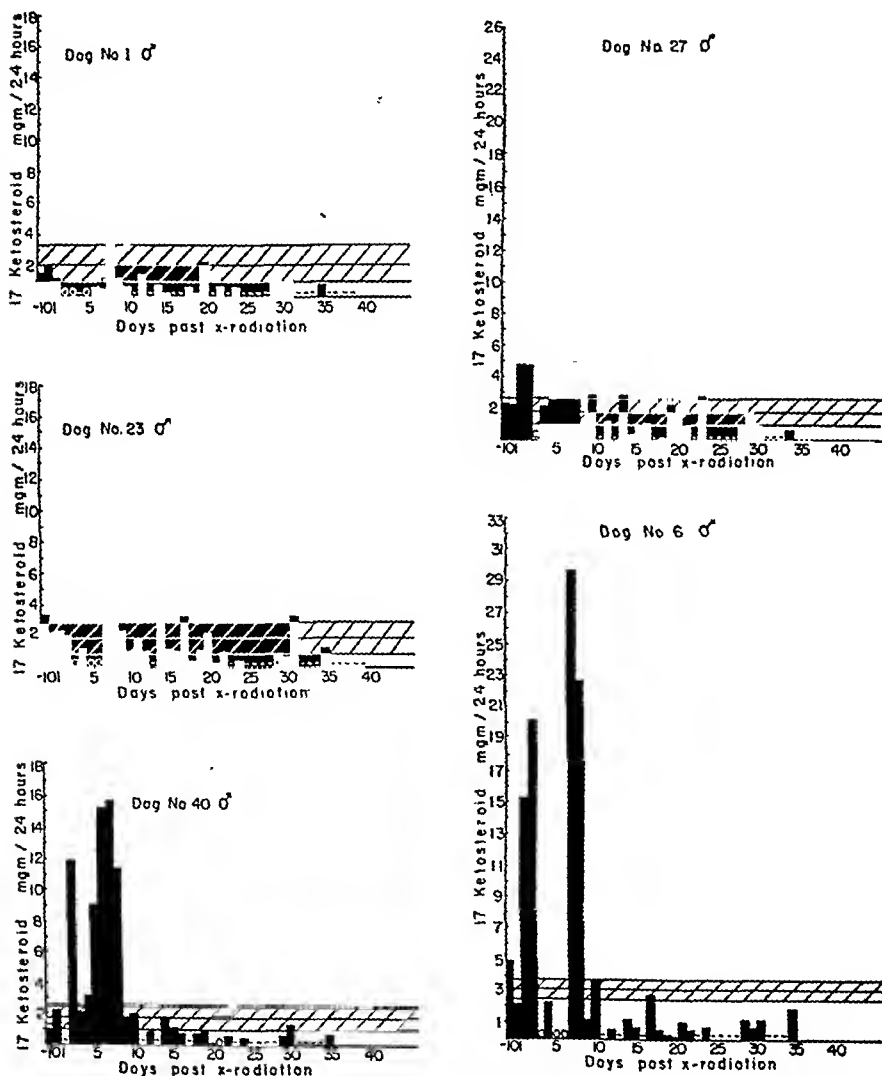


FIG. 1.—Seventeen ketosteroid excretion in X-radiated dogs. The ordinates represent steroid values in mgm. per twenty four hours. The abscissas represent time in days. The shaded areas represent one standard deviation above and below the mean of normal determinations. O indicates no steroid was found in assay. — indicates no assay was made.

As may be observed in figures 1 and 2, eight animals 23, 1, 49, 47, 37, 21, 32, and 50 responded to radiation with an initial increase in 17 ketosteroid excretion which occurred between the fifth to twelfth day following radiation. The increase in all cases was greater than three times the standard deviation and greatly exceeded the normal range. The increased excretion was followed immediately by a fall to normal or subnormal levels and showed a definite trend to the normal range by the thirtieth to fortieth day.

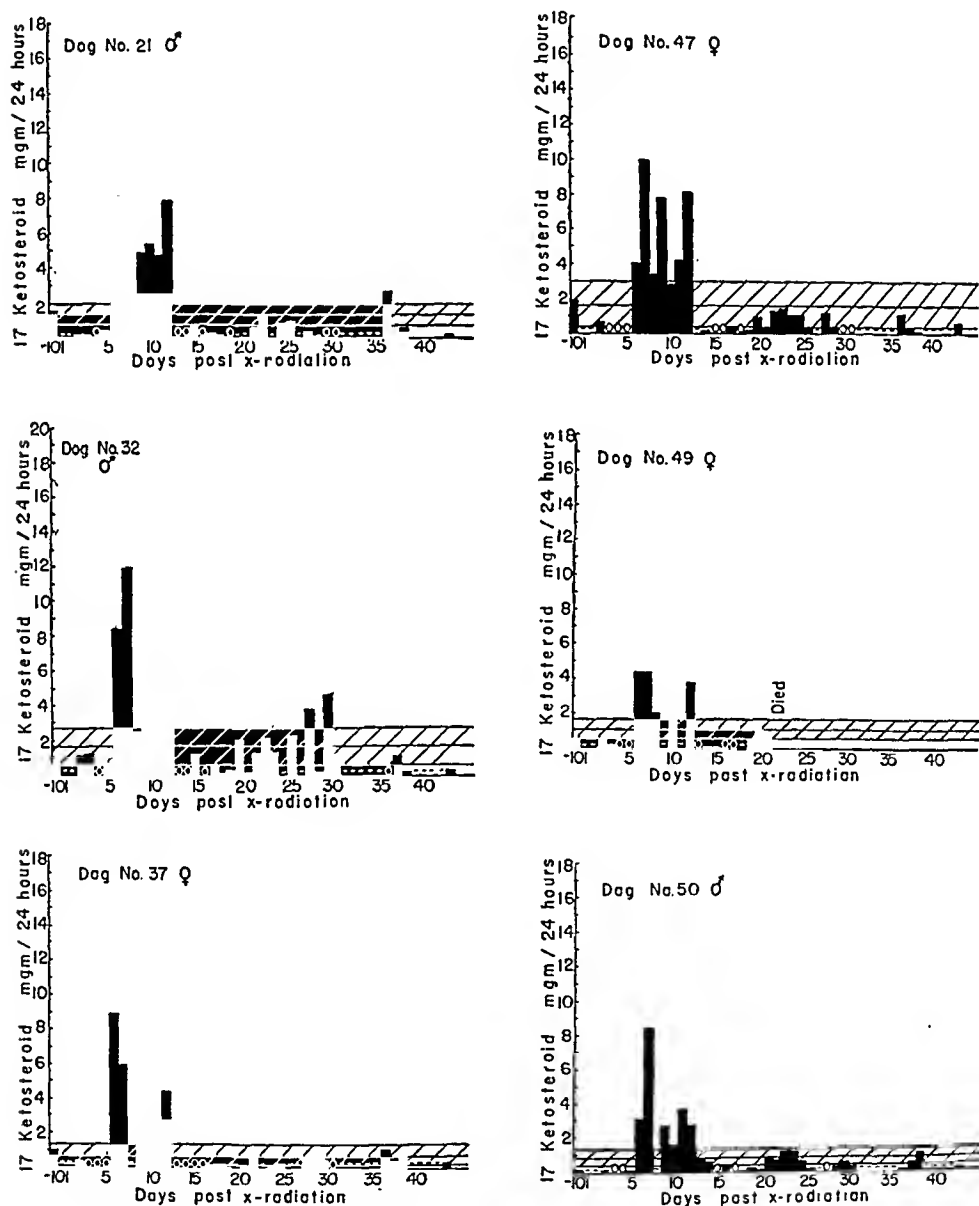


FIG. 2.—Seventeen ketosteroid excretion in X-radiated dogs. The ordinates represent steroid values in mgm. per twenty four hours. The abscissas represent time in days. The shaded areas represent one standard deviation above and below the mean of normal determinations. O indicates no steroid was found in assay. — indicates no assay was made.

In the first group, three male dogs 27, 17, 40 showed a significant rise in 17 ketosteroid excretion on the first and/or the second day following radiation. This was not observed in the second group.

No animals died during the course of the first experiment. One animal, 49, of the second group died on the twenty-first day following radiation. Her rise in 17 ketosteroid excretion although present was never marked.

DISCUSSION

Seventeen ketosteroid urinary excretion values increased in all dogs at some time between the fifth and twelfth day following radiation and then fell to normal or subnormal levels showing a final trend in steroid excretion to normal ranges by the thirtieth to fortieth day. This may be interpreted to indicate an initial period of increased adrenal cortical and/or testicular activity (Fraser, Forbes, Albright, 1941) in response to radiation. This pattern of response agrees with that observed in many cases of stress due to trauma and disease (Cope, *et al.*, 1943; Forbes, *et al.*, 1947; Stevenson, Schenker and Browne, 1944). The time between the stress and the appearance of the rise in 17 ketosteroids is longer in this group of animals than is reported for other forms of stress. Indirect evidence is available in the literature to confirm this delay as peculiar to irradiation. Schwartz (1949) found that several patients exhibited an increase in 11 oxycorticosteroids which occurred on the sixth day following radiation. Patt (1947) found the increase in adrenal cholesterol following LD₅₀ and higher doses of radiation occurred on the sixth to seventh day. X-radiation has recently been reported to produce an initial immediate increase in 17 ketosteroid excretion in patients treated for rheumatoid arthritis. The excretion then fell to levels below the pre-irradiation levels which had been higher than the normals for healthy men and women (Davison, Loets, Kuzell, 1949).

Our findings at this one dosage range appear to be significant due to the uniformity of the excretion pattern. They agree with previous investigations that have illustrated an altered adrenal cortical function in response to X-radiation. The findings would also indicate that irradiation may produce an adaptation response similar to that seen following other stimuli. Assay of other adrenal cortical hormone excretion patterns as well as 17 ketosteroids at different X-ray dose levels will be necessary to establish this concept.

SUMMARY

The urinary excretion pattern of 17 ketosteroids was assayed in 11 dogs following exposure to a minimal lethal dose of total body X-radiation. All animals exhibited a significant increase in steroid excretion at some time between the fifth to twelfth day following radiation. These values then fell to normal or subnormal levels with excretion showing a trend to normal levels by the thirtieth to fortieth

day. This excretion pattern is interpreted to indicate an alteration of adrenal cortical and/or testicular activity in these animals at this dose range.

The hormonal excretion pattern is similar to that reported as occurring in the organisms adaptation to other stimuli. Further studies are necessary to establish this concept.

ACKNOWLEDGMENT

Dr. J. D. Fischer, The Armour Laboratories, Chicago, Illinois, generously supplied adrenocorticotrophic hormone, Lot 41-L, for use in this study. Dr. F. B. Westfall, The National Cancer Institute, Bethesda, Maryland, generously supplied the crystalline dehydroisoandrosterone. F. W. Chambers, Jr., LCDR, MSC, USN, and E. P. Cronkite, LCDR, MC, USN, acted as technical consultants on methods of radiation. J. H. Freeman, HM2, USN, rendered invaluable technical assistance.

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THE EFFECTS OF THYROXIN AND THIOURACIL ON THE TIME OF APPEARANCE OF OSSIFICA- TION CENTERS OF RAT FETUSES

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INTRODUCTION

THE SECRETIONS of the thyroid gland tend to accelerate skeletal ageing while lack of the secretions tends to retard this ageing (Silberberg and Silberberg, 1943, and others). The time of appearance of secondary ossification centers, a stage of skeletal ageing, seems to agree with this concept, for such centers are accelerated in their appearance in rats injected with thyroxin (Noback, Barnett and Kupperman, 1949) and delayed in their appearance in thiouracil-injected rats (Noback, Barnett and Kupperman, 1949), in human cretins (Means, 1937; Caffey, 1945; Albright, 1947; and others), and in thyroidectomized rats (Scow, Becks, Simpson, Asling and Evans 1948).

The purpose of this paper is to consider the relation of the thyroid gland to the time of appearance of primary ossification centers of membrane and cartilage bones (which only appear during prenatal life) in rat fetuses.

MATERIAL AND METHODS

Young adult female albino rats (Sprague-Dawley strain) were mated with males of the same strain. When sperm were identified in the morning vaginal smear or when a vaginal plug was found, the animal was considered to be pregnant as of that morning (Blandau, Boling and Young, 1939).

The experiments were divided into (1) a thyroxin series and (2) a thiouracil series. The distribution of the animals, and the dosage of drugs, in these experiments, is noted in Table 1.

After sacrificing the mothers, the fetuses were fixed in 95% alcohol, cleared and the osseous skeletons stained with alizarin red by the technique of Noback and Noback (1944). An ossification center of a bone was noted as present when stained by alizarin red. Several fetuses from each litter were prepared for observations not in the scope of this paper and hence not included in the number of fetuses observed as noted in Table 1. The thyroid glands of the mothers and the fetuses were fixed in 10% formalin.

The ossification centers examined are among the first to appear in the

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TABLE 1. SUMMARY OF NUMBER OF ANIMALS USED

Experiment	No. of mothers	No. of fetuses* examined for ossification centers	Total No.* of fetuses
<i>Thyroxin Series</i>			
Group I— (animals sacrificed at 15 days of pregnancy)			
Control animals:	4	24	32
Experimental animals: Mothers injected daily with 1 mg. Thyroxin ² from 9th to 15th day of pregnancy.	5	26	37
Group II— (animals sacrificed at 16 days of pregnancy)			
Control animals:	8	53	74
Experimental animals:			
(a) Mothers injected daily with 1 mg. Thyroxin from 10th day through 16th day of pregnancy.	4	22	38
(b) Mothers injected daily with 1 mg. of Thyroxin from 6th to 16th day of pregnancy.	4	24	39
(c) Mothers injected daily with 0.1 mg. of Thyroxin from 3rd to 16th day of pregnancy.	3	33	33
<i>Thiouracil Series</i>			
Control animals: same as in thyroxin series group II.	8	53	74
Experimental animals: Mothers fed with 0.2-0.4% thiouracil ³ from first day of pregnancy until sacrificed.			
(a) Animals sacrificed at 16th day of pregnancy.	10	62	87
(b) Animals sacrificed at 17th day of pregnancy.	4	22	33

* Not all fetuses were examined for ossification centers as several fetuses in each litter were saved for observations not in scope of this paper.

fetus. The degree of development of each center was graded according to (1) a bone index scale and (2) a count of the number of ossified ribs. The bone index is an arbitrary and subjective scale which was devised in an attempt to give some reasonable numerical expression, from a low of 1.0 to a high of 4.0, to the developmental status of the bones observed. The

² The crystalline thyroxin was supplied through the generosity of Dr. C. H. Mann of E. R. Squibb and Sons.

³ The thiouracil (2-thio-6-oxypyrimidine) was supplied through the generosity of Dr. S. N. Hardy and Dr. M. Lockhart of the Lederle Laboratories.

average bone index for each center was computed by averaging all the bone indices of each center for all the animals in each group of experiments. The standard deviation and probable error were calculated for the average bone index of each center. Then the differences between the control bone indices were compared to see if they were statistically significant. The degree of statistical significance of the experimental animals compared to the control animals is tabulated in Table 2.

OBSERVATIONS

Thyroxin Series, Table 1, Figures 1 and 2

The 15 day-old fetuses of the control animals and thyroxine-injected animals had no ossification centers.

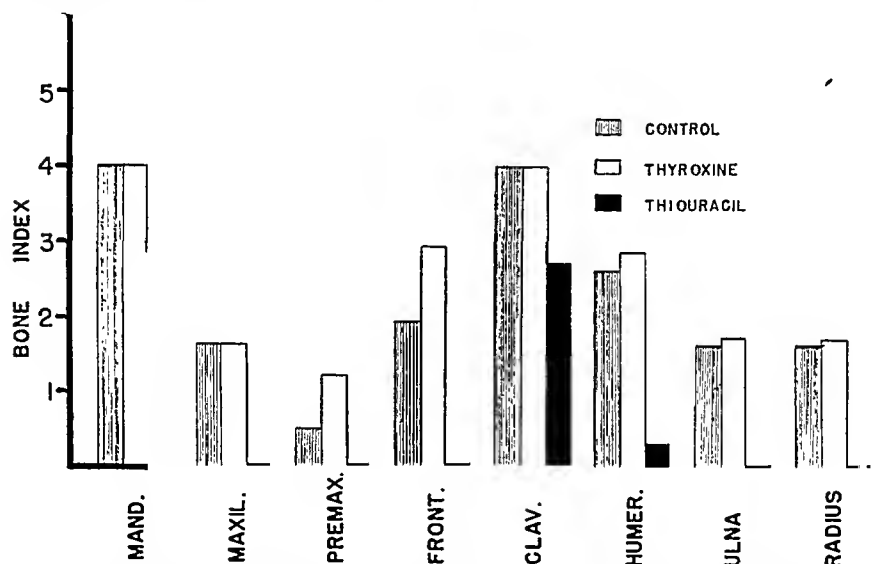


FIG. 1. Bone indices of 16 day-old fetuses from control animals and of those mothers which were injected with thyroxine from the 6th day of pregnancy or fed thiouracil.

The 16 day-old fetuses of the control animals and both series of thyroxine-injected animals had ossification centers for the mandible, maxilla, premaxilla, frontal bone, clavicle, humerus, ulna, radius and ribs. No statistically significant differences were noted between the control animals and any of the series of thyroxine-injected animals, as regards the bone indices and number of ribs.

Thiouracil Series, Table 1, Figures 1 and 2

In this series the 16 day-old control animals are the same animals used in the thyroxine experiment.

The number of ossification centers in the 16 day-old fetuses of females fed the diet containing thiouracil was less than in the control fetuses. Only the mandible, clavicle, humerus, and ribs had ossified,

and the degree of development of their ossification center was greatly reduced as indicated by the bone indices in Figure 1. The number of ossified ribs was 2.1 ± 0.5 in the experimental animals compared to 9.6 ± 0.2 in the control animals. In this experiment there are statistically significant differences between the fetuses of the control animals and of the thiouracil-fed animals as regards the bone indices and number of ossified ribs (Table 2).

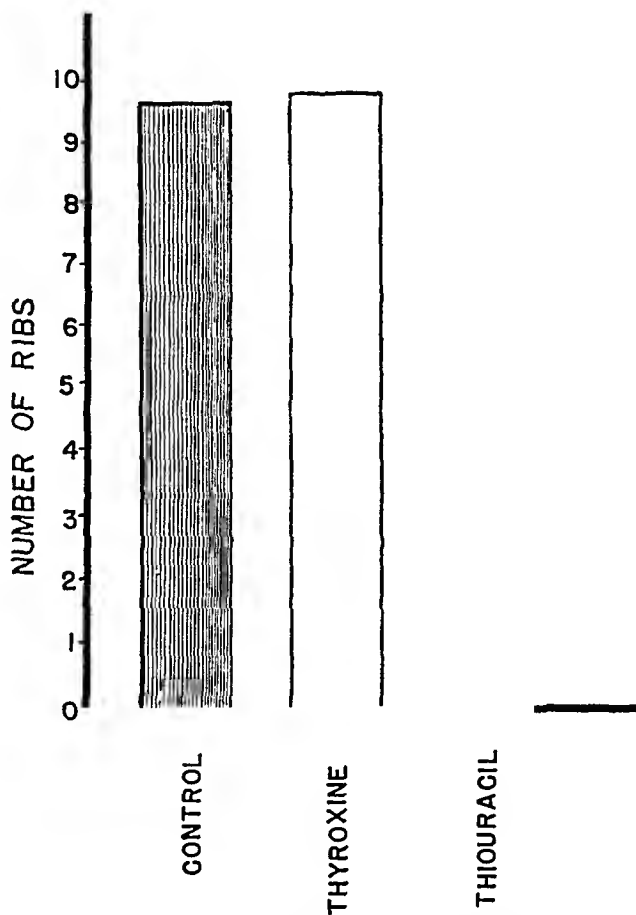


FIG. 2. Number of ribs present in 16 day-old fetuses of animals noted in Fig. 1.

The 17 day-old fetuses of mothers fed thiouracil throughout pregnancy were examined to see if the centers which failed to appear (maxilla, premaxilla, frontal bone, radius and ulna) in the 16 day-old fetuses of thiouracil-fed mothers had appeared. In these 17 day-old fetuses, all of the centers listed above had appeared.

Thyroid Glands of Animals of the Thyroxin and the Thiouracil Series

The maternal thyroid glands showed histological evidence that the thyroxin and the thiouracil were pharmacologically active. The thyroid glands of thyroxin-injected mothers had atrophic acinar cells

TABLE 2. DEGREE OF SIGNIFICANCE (\pm P.E.)* OF DATA OBTAINED FROM FETUSES OF MOTHERS SACRIFICED ON THE 16TH DAY OF PREGNANCY COMPARING CONTROL AND EXPERIMENTAL ANIMALS

Bone	Degree of significance (\pm P.E.)* of data obtained from fetuses of mothers sacrificed on the 16th day of pregnancy		
	Mothers injected with thyroxine from the 10th day of pregnancy	Mothers injected with thyroxine from the 6th day of pregnancy	Mothers fed diet containing thiouracil
Mandible	0.0	0.0	8.6
Maxilla	0.9	0.0	16.0
Premaxilla	1.4	2.3	5.0
Frontal Bone	1.0	2.3	9.5
Clavicle	0.0	0.0	9.3
Humerus	1.4	1.4	10.0
Ulna	0.0	0.7	16.0
Radius	0.0	0.7	16.0
No. of Ribs	0.3	1.4	15.0

* A \pm P.E. greater than 3.0 denotes statistical significance.

and acinar lumina filled with colloid. The hyperplastic glands of thiouracil-fed mothers had hyperplastic acinar cells, most acinar lumina with no colloid, and a few lumina with traces of colloid.

The drugs used apparently had no effect on the fetal thyroid glands, since the differentiating follicles of the fetuses of control, thyroxine-injected and thiouracil-fed animals were microscopically similar.

DISCUSSION

On the basis of the above data it may be concluded that for the dosage of thyroxine injected into the pregnant rats, thyroxine does not significantly influence the time of appearance of the fetal ossification centers under observation. These thyroxine results differ from those of Noback, Barnett and Kupperman (1949) who noted that daily injections of thyroxine into postnatal rats accelerated the appearance of ossification centers appearing 8 to 10 days after birth. The length of time our animals were injected with thyroxine equalled or exceeded the length of time that Noback, Barnett and Kupperman (1949) injected their rats with thyroxine. Thus on the basis of their data the durations of our experiments were sufficient. There are several possible explanations for the difference between the effects of thyroxine on the time of appearance of prenatally appearing centers and postnatally appearing centers. Since evidence in the literature indicates that the thyroid hormone may pass through the placenta of the guinea pig (whose placenta is similar to the rat placenta), and dog (Halstead, 1886; Ukita, 1919; Döderlein, 1928), the differences may not be explained by the lack of any of the injected hormone in the fetus. An explanation may be that the amount of hormone passing through the barrier is insufficient to accelerate the appearance of the ossification centers. It may also be that the fetus is under maximal thyroid sti-

mulation from the mother in the normal pregnant rat and is refractory to further thyroxin stimulation.

As regards the thiouracil experiments, it may be concluded that thiouracil delays the time of appearance of the fetal ossification centers examined. Since thiouracil inhibits the production of endogenous thyroid hormone (Astwood, 1949), it may be inferred that the delay in the appearance of the centers is probably due to thyroid hormone insufficiency. Compensatory fetal thyroid activity is inhibited since thiouracil passes through rat placental barriers (Freisleben and Kjervuld-Jansen, 1946). This is similar to the observations on the delay of the appearance of secondary ossification centers in human cretins (Albright, 1947; Means, 1937; Caffey, 1947, and others), in thyroidectomized rats (Scow *et al.*, 1948), and in thiouracil-injected rats (Noback, Barnett and Kupperman, 1949).

Our data may indicate that the maternal thyroid gland secretions have a role in the differentiation of the ossification centers studied in the 16 day-old fetus. This is based on the assumption that the evidence in the literature is conclusive in indicating that the fetal rat thyroid gland does not become active until the 17th to 19th day of pregnancy. Colloid in the thyroid follicles is first seen in 17 day fetal rats⁴ (Hall and Kaan, 1942). No follicular colloid is present in any of the 16 day-old rats in our series. The 19 day-old fetal rat⁴ thyroid gland shows physiological activity as measured by the effects of the injection of these glands on tadpole metamorphosis (Hall and Kaan, 1942). Maternally-injected radio-active iodine is first concentrated in the thyroid gland of the 18 to 19 day-old rat fetus (Gorbman and Evans, 1941). If the rat fetal thyroid gland does not secrete thyroid hormone by the 16th day of pregnancy, then the fact that the centers of ossification were delayed in their appearance in the 16 day-old fetuses of mothers injected with thiouracil suggests that the maternal thyroid hormone has a role in the time of appearance of the ossification centers studied.

These results indicate that the thyroid gland has a role in the differentiation of membrane bones (frontal bone, etc.) and of primary endochondral bones (humerus, etc.).

SUMMARY

The time of appearance of the ossification centers studied was not significantly altered in the 16 day-old rat fetuses the mothers of which were injected daily with 1 mg. of crystalline thyroxin from either the 6th or the 10th day after pregnancy.

The time of appearance of the same ossification centers was de-

⁴ To make the fetal ages comparable we are adding one day to Hall and Kaan's (1942) ages since these authors calculated fetal age commencing one day after sperm were observed in the vaginal smears while our calculations commenced on the day sperms were observed in the vaginal smears.

finitely delayed in the 16-day-old rat fetuses the mothers of which were fed on a diet containing 0.2 to 0.4% thiouracil from date of pregnancy.

On the basis of these results it may be concluded that the thyroid gland has a role in influencing the time of appearance (differentiation) of the ossification centers of membrane bones and primary endochondral bones. Furthermore, it is suggested that thyroid hormone from the maternal thyroid gland may be essential for normal differentiation of the observed ossification centers appearing in 16 day-old rat fetuses.

ACKNOWLEDGMENTS

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THE METABOLIC RATE AND THYROID SIZE OF CHICKS FROM DAMS WITH ALTERED METABOLISM¹

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THE EFFECT of the thyroid hormone and synthetic thyroxine or thyroprotein and goiterogenic substances, such as thiouracil, on the metabolic rate and thyroid size of experimental animals has been well established. Relatively little information is available, however, on the effect of hyperthyroidism or hypothyroidism of the dam on the metabolic rate of her progeny.

Hens fed a ration containing thyroprotein have been shown to produce chicks with enlarged or goiterous thyroid glands (Wheeler and Hoffman, 1948a, and McCartney and Shaffner, 1949). Chicks with goiterous thyroids have also been shown by Andrews and Schnetzler (1945) to be produced by females fed thiouracil in the ration.

Since the chicks from thyroprotein-fed hens had a prolonged incubation period, a significantly greater thyroid size, and a lowered heart rate, Wheeler and Hoffman (1948a) concluded that they were hypothyroid. Assay of chick thyroidal activity, as determined by the closed-vessel technique of Smith et al. (1947), led McCartney and Shaffner (1949) to believe that the metabolism of chicks produced by both thyroprotein-fed and thiouracil-fed females is lower than that of normal chicks, suggesting a reduced metabolic rate and a functional hypothyroid condition.

The present experiment was undertaken to further investigate these unexpected observations and to study the mode of action of the compounds and the post-natal duration of the effects.

METHOD

On September 1, 1948, sixty New Hampshire pullets were selected and placed in individual laying cages. After four weeks, these females were divided at random into three groups of twenty birds each. Thyroprotein³ was added to the ration of one group at a level of 0.022 per cent and thioura-

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³ The thyroprotein used in this experiment, called Protamone, was kindly supplied by Dr. W. R. Graham, Jr., of the Cerophyl Laboratories, Inc., Kansas City, Missouri.

cil⁴ to the ration of a second group at a level of 0.1 per cent. The third group served as controls.

The resting metabolism of these females was determined at 4-week intervals by means of the indirect calorimetry method, employing a closed circuit type of respiratory apparatus. All metabolism tests were run at night between 6 P.M. and 12 P.M. after the birds had been fasted for approximately twelve hours. All measurements were recorded on the basis of cubic centimeters of oxygen consumed per kilogram of body weight per hour, corrected to standard temperature and pressure.

In order to measure the thyroidal activity of the day-old chicks produced by the different groups of females, the oxygen consumption of groups of 18 chicks each, maintained at 32° to 35°C., was measured. The method used to obtain these determinations was the same as that employed to estimate the resting metabolism of their dams. A series of eight observations were made on chicks produced by each of the three different groups of females over a period of 12 weeks.

To determine the rate at which the chick's thyroid gland size was affected by feeding its dam thiouracil or thyroprotein, groups of day-old chicks (10 per group) were sacrificed at weekly intervals during the treatment period and their thyroid glands weighed. These observations were made beginning with the first hatch after the initiation of treatment and continued for the next ten successive weekly hatches. All thyroids were freshly dissected and weighed on a Roller-Smith balance to the nearest 0.1 milligram.

To study the effect of these treatments on the post-natal changes in thyroid size, chicks produced by the three groups of females were reared in electric battery-brooders with raised screen floors and were fed a conventional all-mash ration. Samples of 10 chicks at day-old and at 7, 14, and 21 days of age, respectively, were sacrificed, the thyroid glands removed and weighed to the nearest 0.1 milligram.

In studying the effect of exogenous thyroxine on the thyroid size of the chick, eggs from control females were injected with aqueous solutions of thyroxine or thyroprotein by puncturing a small hole in the shell over the air cell and applying the solution by means of a tuberculin syringe on the inner shell membrane. Following the injections, the holes were sealed with paraffin wax and the eggs placed in the incubator. Thyroxine was injected at levels of 6, 12, 20, and 40 micrograms and thyroprotein at levels equivalent to 9, 12, 20, and 40 micrograms of d,l-thyroxine. Similarly, eggs produced by thyroprotein-fed females were also injected with thyroxine, at levels of 2, 4, 6, 8, and 10 micrograms.

Since iodized casein preparations have been shown to contain from 7.7 to 8.7 per cent iodine (Harrington and Pitt Rivers, 1939), it was thought that possibly excess amounts of iodine may have been transmitted to the eggs produced by the thyroprotein-fed females, producing chicks with enlarged thyroids. Therefore, iodine in the form of KI was injected into control eggs at levels of 4, 6, 8, 10, 20, and 40 micrograms, respectively. Day-old chicks hatched from the injected eggs were sacrificed and the thyroid glands weighed to the nearest 0.1 milligram. Approximately 500 eggs were used in the thyroxine, thyroprotein, and KI injection studies.

⁴ The thiouracil (Deravet) was supplied through the courtesy of the Lederle Laboratories, Pearl River, New York.

RESULTS

The average oxygen consumption of the control females and of the females receiving thyroprotein or thiouracil in their ration is shown in Table 1. These data indicate that the addition of 0.022 per cent thyroprotein to the ration brought about a small but highly significant increase in the rate of resting metabolism. After 12 weeks of treatment the average rate of resting metabolism of these females was about 12 per cent more than that of the control females. Feeding 0.1 per cent thiouracil in the ration caused a marked decrease in the rate of metabolism. The average rate of resting metabolism of these females was approximately 24 per cent less than that of the controls. This difference in oxygen consumption of 204 ± 30 cc. per kilogram of body weight per hour between the control females and the females fed thiouracil for 12 weeks is highly significant statistically. These results provide sufficient evidence to indicate that mild hyperthyroidism and hypothyroidism were experimentally induced in the females receiving thyroprotein and thiouracil, respectively, in their ration.

TABLE 1. RESTING METABOLISM OF CONTROL FEMALES AND FEMALES FED THYROPROTEIN OR THIOURACIL

Weeks of treatment	Cc. O ₂ /kilo/hour*		
	Control	Thyroprotein	Thiouracil
0	934 \pm 39	—	—
4	930 \pm 27	982 \pm 47	837 \pm 31†
8	798 \pm 20	1012 \pm 35†	716 \pm 24†
12	843 \pm 25	943 \pm 32†	639 \pm 18†

* Cubic centimeters of oxygen consumed per kilogram of body weight per hour.

† The difference between these values and the control is highly significant.

The average oxygen consumption, in milliliters per kilogram of body weight per hour was 1981 ± 74 cc. in chicks from the control females, 1660 ± 115 cc. in chicks from the females receiving thyroprotein, and 1644 ± 90 cc. in chicks from the thiouracil-fed females. The chick-metabolism tests were made during the last half of the 12-week treatment period of their dams. The chicks produced by the thyroprotein-fed and the thiouracil-fed females consumed 16.2 and 16.5 per cent, respectively, less oxygen than the control chicks. In each case, the decrease in oxygen consumption of the chicks from the treated dams is statistically significant.

In Figure 1 are presented results showing the progressive change in chick thyroid size following initiation of treatment to their dams. The results of this study indicate that the thyroid glands of the chicks produced by the thiouracil-fed females had not attained their maximum size after 11 weeks of treatment, while the thyroids of the chicks produced by the thyroprotein-fed females had apparently reached their greatest size (about 11.0 milligrams) after 8 weeks of treatment.

The lines shown in Figure 2 represent the change occurring in the

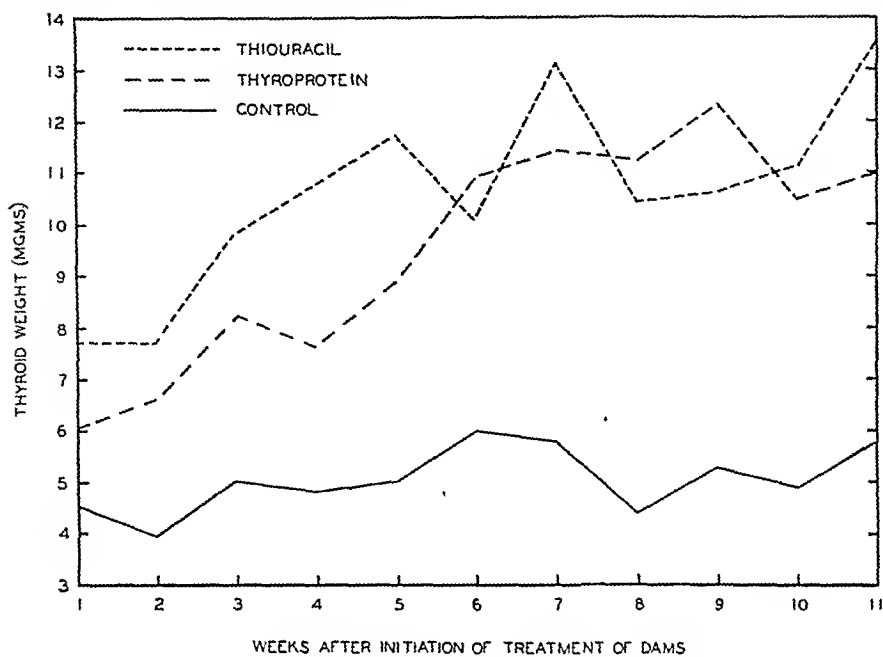


FIG. 1.—Thyroid weight of day-old chicks from control dams and dams fed thiouracil or thyroprotein.

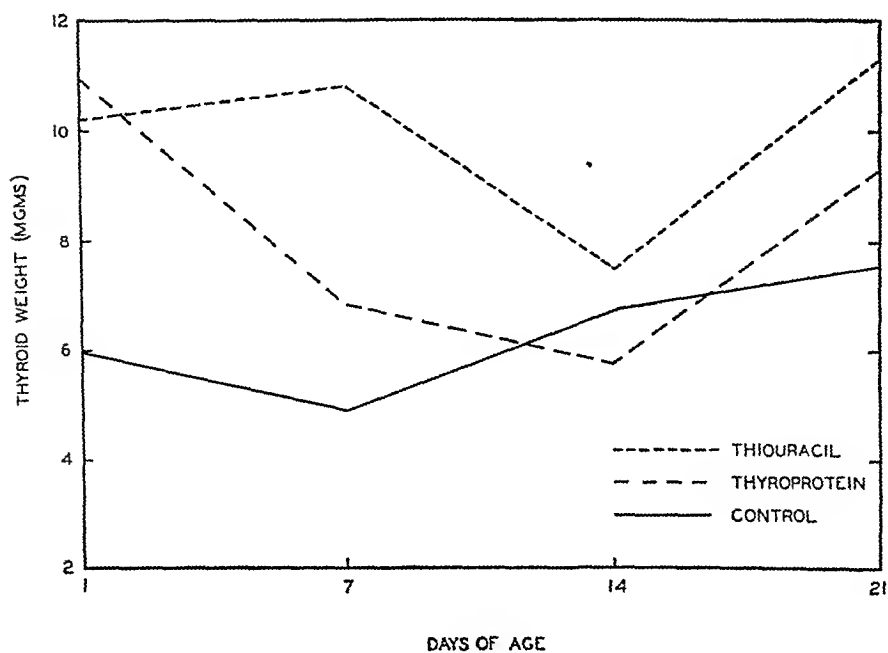


FIG. 2.—Change in thyroid weight of chicks produced by control dams fed thiouracil or thyroprotein (day-old to 21 days of age).

thyroid weight from day-old to three weeks of age of chicks produced by the three groups of females. The thyroid weights of the chicks from the thyroprotein-fed females decreased during the first week, while those of the chicks from the thiouracil-fed females increased somewhat. By the end of the second week the thyroid glands of the chicks from the treated dams were comparable in size with those of the controls. The thyroids of the three groups of chicks increased slightly during the third week of the post-hatching period.

McCartney and Shaffner (1949) have previously shown that chicks with enlarged thyroids were produced by females fed a ration containing thyroprotein or thiouracil, while a decrease in thyroid size resulted when thyroxine was injected into control eggs. The thyroid glands of the chicks hatched from eggs injected with 6, 12, 20, and 40 micrograms, respectively, of d,l-thyroxine were consistently smaller than the thyroids of normal chicks. Preliminary studies with thyroprotein-injected eggs (9 and 12 micrograms) were too limited to determine its effects on thyroid size. However, subsequent studies revealed that thyroprotein injected into control eggs at levels equivalent to 20 and 40 micrograms of d,l-thyroxine had no effect upon chick thyroid size.

Since chicks with goitrous thyroids are produced by thyroprotein-fed females, eggs laid by these females were injected with thyroxine at levels of 2, 4, 6, 8, and 10 micrograms. The results of these thyroxine-injection studies revealed that approximately 6 micrograms of exogenous thyroxine is necessary to affect thyroids of normal size in chicks produced by thyroprotein-fed females.

The thyroid glands of chicks hatched from control eggs injected with KI at levels as high as 40 micrograms were comparable in size with the thyroids of normal chicks.

DISCUSSION

The results obtained on the rate of resting metabolism indicate that mild hyperthyroidism and hypothyroidism were experimentally induced in the females fed 0.022 per cent thyroprotein and 0.1 per cent thiouracil, respectively, in their ration. The rate of oxygen consumption of the thyroprotein-fed females, after 12 weeks of treatment, was 12 per cent more than that of the control females. This increase in metabolism is comparable with the changes in metabolism obtained in other animals by feeding thyroprotein. The decrease of approximately 30 per cent in the resting metabolism of the thiouracil-fed female chickens during a period of 12 weeks is somewhat less than that reported by Shaffner and Andrews (1948) for male chickens during a similar period of time, indicating that there may be a sex difference in response to thiouracil.

Day-old chicks produced by each of the two groups of treated females required about 16 per cent less oxygen than the control chicks. Since these resting metabolism results are in fairly close agree-

ment with the survival-time results reported by McCartney and Shaffner (1949), it seems that there is little doubt that the chicks produced by both the hyperthyroid and the hypothyroid dams are themselves hypothyroid.

According to Wheeler and Hoffman (1948b), the thyroid enlargement of chicks from thyroprotein-fed hens is maximum about 14 days after the initiation of treatment. These results are not in accord with the findings reported herein, since in this experiment chick thyroid enlargement did not reach a maximum (greatest size of approximately 11.0 milligrams) until 8 weeks after the females were put on the experimental ration. If chicks with enlarged thyroids are the result of a decreased secretion of the maternal thyroid hormone in females fed thyroprotein, this indicates that the maximum reduction in deposition of maternal thyroxine was not reached until several weeks after the exogenous thyroxine was administered in the ration. However, chicks with goitrous thyroids occurred concurrently with the addition of thyroprotein to the ration of their dams, the thyroids being significantly larger within one week from the beginning of treatment.

The thyroid glands of the chicks produced by the thiouracil-fed females were still increasing in size after eleven weeks of treatment. This progressive increase in thyroid size would indicate that more thiouracil and/or less thyroxine was transmitted from the female to her eggs as the treatment advanced. Since the resting metabolism of these females decreased progressively during the experimental period, it is apparent that a relationship exists between the degree of hypothyroidism of the female and the amount of thyroid enlargement of her chicks.

Since the thyroid glands of the chicks from thiouracil-fed dams continued to enlarge during the first week after hatching, it would appear that thiouracil was stored in the chick's yolk sac. The thyroid weight of the chicks produced by the thyroprotein-fed females decreased during the first week, suggesting that there is little or no carry over in the yolk sac of the factor or factors causing thyroid enlargement in these chicks.

Thyroprotein injected into control eggs at levels as high as 40 micrograms of d,1-thyroxine had no effect on chick thyroid size, indicating that the thyroprotein cannot be assimilated or utilized by the developing chick embryo. It seems logical to assume that the thyroxine in a solution of thyroprotein should pass through the shell membrane as readily as a solution of synthetic thyroxine. Booker and Sturkie (1949) injected thyroprotein into the albumen of incubating eggs at a level of 33 gamma (equivalent to 1 gamma of d,1-thyroxine) and obtained a slight decrease in thyroid size. However, in view of the limited number of observations cited, the difference between the thyroid size of the chicks from the control eggs and the injected eggs could have been easily due to sex. Males have significantly smaller thyroids than females (Aberle and Landauer, 1935).

The injections of thyroxine into eggs laid by the thyroprotein-fed females consistently reduced thyroid size, indicating that there was actually a deficiency of thyroxine in these eggs. Approximately 6 micrograms of exogenous thyroxine was necessary to affect thyroid glands of normal size in chicks from eggs produced by females receiving 0.022 per cent thyroprotein in the ration.

The results of the KI injections indicate that excess iodine transmitted from the dam to her eggs was not responsible for causing chicks with enlarged thyroids. Since a laying hen consumes about one-quarter of a pound of feed daily, it was assumed that the daily intake of iodine in thyroprotein was somewhere within the limits of 300 and 400 micrograms. Since only a small per cent of the total iodine intake of the thyroprotein-fed females could possibly be transmitted to their eggs, it seems probable that 40 micrograms of injected KI would be comparable to the maximum amount of iodine that could be transmitted from these dams to their eggs. Albert et al. (1946) injected groups of day-old cockerels (10 to 20 per group) with solutions of KI and has shown that KI injected at levels as high as 10 milligrams per chick had no effect on thyroid size.

SUMMARY

Three groups of New Hampshire females, 20 per group, were each maintained on a control ration, a ration containing 0.022 per cent thyroprotein, and a ration containing 0.1 per cent thiouracil, respectively. After 12 weeks the resting metabolism of the thyroprotein-fed and the thiouracil-fed females was 12 per cent more and 24 per cent less, respectively, than that of the control females.

Day-old chicks produced by each of the two groups of treated females required about 16 per cent less oxygen than the control chicks. The results of the metabolism studies indicate that chicks produced by both hyperthyroid and hypothyroid females are themselves hypothyroid.

About 6 micrograms of exogenous thyroxine were necessary to affect thyroids of normal size in chicks hatched from eggs laid by thyroprotein-fed females. Aqueous solutions of thyroprotein or KI injected into control eggs had no effect on chick thyroid size.

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THE EFFECTS OF STRESS, ADRENAL AND ADRENOCORTICOTROPHIC HORMONES ON THE CIRCULATING EOSINOPHILS OF MICE¹

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THE EOSINOPHIL cell of the blood was first described over 100 years ago (Wharton Jones, 1846), and since then has been the subject of many morphological, cytological, and physiological investigations. However, despite the vast array of publications and reviews, the actual function of this cell in health or disease still eludes the investigator (Kirk, 1942; Bethell, Sturgis, Rundles and Meyers, 1946). A number of comprehensive studies have been made during the last 17 years but most of the investigations were carried out either in man or the guinea pig. Work in other animals seems to have been chiefly of a tabulative and descriptive nature (Scarborough, 1930).

In man, an increase in the number of circulating eosinophils has been correlated with allergy, parasitic infection, skin diseases, and a number of other disorders. In the guinea pig the eosinophils are associated with foreign protein reactions such as anaphylaxis and immunity. (For extensive references and reviews see: Schwarz, 1914; Ringeon, 1938; Rud, 1947; and Samter, 1949.)

The factors involved in decreasing the number of eosinophils seem to be alike in at least one respect, they all produce a stress. In 1939 Dalton and Selye pointed out in the rat that the eosinophils first decreased in number and then increased after various alarming stimuli. Selye (1947) later included this pattern of response in his concept of the general adaptation syndrome. More recently Hills, Forsham and Finch (1948) demonstrated that ACTH and Compound F produced a consistent decrease in the number of circulating eosinophils. They also reviewed the literature briefly and noted that eosinopenia follows various stress stimuli such as hemorrhage, cold, hemolysis, poisons,

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operative procedures, and a wide variety of infections. At the same time Forsham, Thorn, Prunty and Hills (1948) incorporated this decrease of eosinophils after ACTH injections into a clinical test to determine whether the adrenal cortex was functioning normally. These experiments indicate that a decrease in the number of circulating eosinophils is associated with an increased adrenal cortical activity.

The greatest handicap in working with eosinophils is the relatively small number of cells found in normal blood. Using the blood film method, large numbers of cells must be counted in order to determine accurately the percentage of eosinophils. However, in clinical work, this was overcome to some degree by Dunger who in 1910 reported a diluent for blood which stains the eosinophils and destroys the other blood cells. This was based upon the known fact that the eosinophil is the most resistant cell of the blood, especially to many hypotonic and acetone solutions. Since 1910 many workers have used the Dunger or similar procedures. Recently Rud (1947) improved this diluent and applied extensive statistical analyses to his techniques and results. He pointed out that the direct chamber method is superior to the blood film method when quantitative variation of the eosinophil is to be determined. Discombe (1946) agrees with this and states that the uncertainty of the hemocytometer count varies with the square root of the number of eosinophils counted. Randolph (1944) claimed that propylene glycol is superior to acetone as a diluent for human blood.

The authors are not aware of any specific eosinophil diluent devised for use in animal studies, although a number of vital dyes have been used to study the blood picture as a whole (Cunningham and Tompkins, 1938). Preliminary investigations in the mouse and rat had indicated that the eosinophils respond specifically and quantitatively to adrenal cortical compounds. It seemed advisable therefore to work out an eosinophil diluent which could be used in rodents. The following paper consists of a detailed account of the general techniques used for the determination of eosinophil cells in mice and rats, and the application of these techniques to a study of eosinophil numbers as influenced by various factors. A second paper is now being written which will describe a suggested procedure for determining quantitatively the amount of adrenal cortical hormone in various extracts and preparations.

MATERIALS AND METHODS

Two general techniques have been used in these studies. The first method is a slight modification of the standard clinical procedures (Lillie, 1948). Total white blood cell counts are obtained by diluting the blood in a 4% acetic acid solution. Differential counts were made from blood films prepared as follows: Thin smears are made on slides and fixed for 15 minutes in absolute methyl alcohol. The slides are then placed in a coplin jar and held

approximately 5 millimeters off the bottom of the jar to permit circulation of fluid beneath and between the slides. 40 cc. of diluent³ is poured over the slides and then by use of a medicine dropper, 1½ cc. of concentrated stain⁴ is mixed with the diluent. Care must be taken to avoid direct contact of the concentrated stain with the film side of the slide and to mix the concentrated stain as quickly as possible with the diluent. Occasional agitation is necessary during the staining process. The staining time varies from 2 to 8 minutes depending upon the density of stain desired and the type of blood used. The stain can be rapidly flooded out of the coplin jar with tap water, or the slides removed one at a time and rinsed quickly under the tap.

When the staining time is short, the eosinophil cells contain large reddish granules and very pale blue chromatin; and as the staining time is increased the chromatin stains more intensely. The neutrophil cytoplasm remains colorless and contains extremely minute granules; the lymphocyte cytoplasm stains a blue color.

The above procedure was found to have definite advantages over the standard methods for staining blood, especially mouse blood. The eosinophil granules were brought out much more distinctly. As many as 8 slides could be stained at once, and consistent results obtained from slide to slide. No precipitate forms on the slides.

Differential counts of at least 400 cells were made. To simplify the procedure we divided the white blood cells into 3 types:

1. Mononuclear cells, which include lymphocytes and monocytes.
2. Polymorphonuclear cells, which include neutrophils and all other myelogenous cells except eosinophils.
3. Eosinophil cells, which are cells containing numerous large reddish granules in the cytoplasm. The nuclei are usually bilobed or annular.

The absolute number of each type of cell was determined by multiplying the per cent of each cell in the differential count by the total number of blood cells.

However, it soon became evident that if 50 or more determinations of eosinophils were going to be made per day, that the procedure of total and differential counts took too much time. A survey was made of the various methods of determining eosinophils, but none was found applicable to the mouse or rat with any degree of accuracy. An experimental study was undertaken, therefore, to determine whether it was possible to obtain a diluting solution which would destroy the red blood cells and all the white blood cells except the eosinophils. Numerous water and mild alkaline solutions do this but the fragility of the eosinophils is increased so that any shaking of the diluting pipette causes them to disintegrate rapidly. Acetone was found to be more efficient in preventing the breakdown than any other substance used.

³ Diluent consists of:

680 cc. Distilled Water
120 cc. Acetone
200 cc Buffer at pH 7.3'
(McIlvaine's or Sorenson's)

⁴ Concentrated Stain consists of:

1 gram powdered Wright's Blood Stain
1 gram powdered Giemsa's Blood Stain
75 cc. Diethylene Glycol
25 cc. Glycerine

In very dilute solutions (less than 5% in water) lysis of all cells occurred especially after shaking. As the concentration of the acetone was increased to 25%, more and more of the blood cells remained intact. A 15% acetone solution was found to give the best results in mice and rats. At this concentration the eosinophils remained intact with distinct cell membranes, while the other white blood cells formed hazy images in the background. The red cells were almost completely destroyed.

Phloxine was found to be a good stain for bringing out the eosinophil granules in the acetone solution. A small amount of detergent was added to aid in obtaining a better distribution of cells. Diethylene glycol was added to increase the viscosity of the solution and in some instances it was found to increase the total number of remaining eosinophils. The following formula was found most satisfactory in the mouse and rat:

- 10 cc. 0.1% Phloxine Solution
- 1 cc. Diethylene Glycol
- 4 drops 0.5% Alconox (Detergent)
- 30 cc. Distilled Water.
- Just Before Using Add:
- 7 cc. Acetone

The eosinophils are recognized as follows: the granules within the cell stand out as distinct red bodies; they may be in a mass in the center of the cell, or scattered around the periphery. Usually the cell outline is seen only faintly, and the nucleus is completely colorless. The rat eosinophil is much larger and stains more deeply than the mouse eosinophil.

The procedure used to obtain blood from mice and rats is very important. Great variation in the number of eosinophils will be obtained for the same animal from one pipette to another unless a very highly standardized procedure is followed. For this reason, we include here in considerable detail the procedure we have found most satisfactory for obtaining a direct eosinophil count in mice. Slight modifications of the same procedure will give satisfactory results in rats.

Materials needed for a direct eosinophil determination:

- WBC diluting pipettes and rubber aspirators
- Fuchs' Rosenthal Counting Chambers, 0.2 mm depth
- Microscope, preferably bifocal with 15X wide field oculars and 16 mm. objective.
- Thin new razor blades for cutting tail
- Cheese cloth for holding animals
- Cleansing tissues
- Eosinophil diluting fluid

Procedure for obtaining a blood sample in the mouse:

1. The mice are heated for approximately 10 minutes under a 100 watt lamp. This is best done in a battery jar containing about 1 inch of wood chips at the bottom. The mice should become active so that the blood circulation in the tail is stimulated.
2. A mouse is then wrapped loosely in cheesecloth with the tail projecting out, and held firmly in the palm of one hand. The tail is washed with soap and warm water.
3. With a sharp razor a small nick is made through the skin, then through one of the prominent blood vessels just beneath the skin. If properly

done, large drops of blood will quickly form over the cut, and the tail will later heal over without showing any deformity.

4. The first two drops of blood are discarded and the wound and surrounding area carefully wiped with a cleansing tissue to remove any debris and blood. Fresh blood is then steadily drawn into the WBC pipette as it oozes from the wound until it reaches the 0.5 mark on the pipette. Avoid taking blood that has been exposed to air for more than a few seconds, or which shows any clot formation.
5. The eosinophil diluent is quickly drawn into the pipette as the pipette is rotated between the fingers to facilitate mixing of blood and diluent. Fill to the 11 mark and shake gently by hand for 5 to 10 seconds. Fill the counting chamber immediately, discarding the first 3 drops and placing the 4th and 6th drop one on each side of the chamber.
6. Allow the counting chamber to stand for at least 3 minutes and carefully count the number of cells in each chamber. The number of cells in 16 squares (one side) multiplied by 6.25 equal the number of cells per cubic millimeter of blood.

The acetone prevents the breaking down of the white blood cells. Therefore, if the eosinophils appear broken and the granules to be spreading, add more acetone to the diluent. If the other white blood cells remain and hinder the recognition of the eosinophils, decrease the amount of acetone in the diluent. If the granules are not stained deeply enough, allow the chamber to stand for a few minutes longer before counting. If still lightly stained, decrease the acetone in the diluent. It is usually better to remake the eosinophil diluent every time it is used. We keep stock solutions on hand which can be mixed quickly, although on occasion acetone can be added to old diluent and satisfactory counts obtained.

It takes some experience and practice to make accurate determinations of the numbers of eosinophils by the direct method. It is to be expected that the cells in the hemocytometer chamber will be distributed in a Poisson fashion and this has been observed by numerous workers (Discombe, 1946; Rud, 1947). If the cells are distributed in this manner the variance should be equal to the mean number of cells counted. This was checked by making two duplicate counts from each of 60 pipettings covering a wide range of eosinophil concentrations. The value of χ^2 turned out to be 60.79 which for 60 degrees of freedom corresponds to a probability of .45, indicating satisfactory agreement of the data with the Poisson hypothesis.

However, there are errors other than those of the counting chamber in an estimation of eosinophils by the direct method. For the purpose of determining the over-all error, including that which might be due to variation in eosinophil concentrations in different drops of blood from the same animal, counts were made on four successive drops of blood from each of 20 animals. As was to be expected, the variance was greater than that of the counting chamber alone, but there was still proportionality between the variance and the mean number of cells counted, or between the standard deviation and the square root of the number counted. For this series, the ratio of the standard deviation to the mean number of cells counted averaged 1.70. If this figure is taken at face value, one can say that the standard deviation of a given cell count is about 1.7 times the square root of the actual number of cells enumerated.

It should be emphasized that the value of 1.7 was obtained from only

80 counts on 20 animals and that more data may require that this figure be adjusted. Improvements in technique probably would lower the error somewhat, although it could never be less than one times the square root of the number of cells counted. However, this provides an approximate estimate of the magnitude of error of a single count and shows that this is much less than that of ordinary differential counts (Rud, 1947). The error can be further reduced, if need be, by taking duplicate counts from the same animal.

Error of a greater magnitude seems to occur in the counts of blood from those animals which are not sufficiently active to produce good circulation of blood in their tails. This is particularly true if the tail has to be milked to obtain enough blood. Furthermore sick animals that do not become active on heating very often yield successive drops of blood which show great variation. On the other hand if too much heat is applied, the animals may become overactive and then go into a state of collapse.

In our experience the direct method described above when performed by experienced technicians is quicker, easier, and more reliable. This conforms with the published reports of Randolph (1944), Discombe (1946) and Rud (1947), who have used similar direct methods in the clinic. In general the film procedure is used to determine over-all blood changes following the injection of material having unknown physiological action. The direct method is used for the detection of quantitative variations of the eosinophils following different doses of the material.

The mice used in these experiments were C 57 Brown mice obtained from the R. B. Jackson Memorial Laboratory, Bar Harbor, Maine. Adult male mice weighing over 20 grams were found to give the most consistent results and were also the easiest to adrenalectomize. The animals were maintained on an enriched laboratory diet for one week after being received. After adrenalectomy or hypophysectomy a 1% saline solution replaced their drinking water and additional sugar was mixed in their food or placed in separate dishes.

Most of the rats were obtained from Holtzman Laboratory Animals, Inc., Madison, Wisconsin, and weighed from 100 to 200 grams.

OPERATIVE TECHNIQUES

The various operative techniques used in the mouse are very similar to those used in the rat and described by Ingle and Griffith (1942). Our technique of hypophysectomy is a modification of a procedure described by Thomas (1938). In most cases the whole operation can be performed in less than 10 minutes, with an extremely low mortality. In over 200 operations performed over a period of three years, very few of the mice ever showed an increase in weight after the operation.

The adrenalectomies can likewise be performed with little or no mortality in a one stage operation lasting about 2 minutes. The right adrenal is sometimes difficult to remove without rupturing and pieces of the capsule are often left in the body cavity. For this reason all animals must be tested first by a procedure to be described in this paper, before they can be assumed to be completely adrenalectomized.

PROCEDURE AND RESULTS

A. Eosinophil determinations in normal mice

The eosinophil count of mice as well as other animals varies

greatly from time to time depending upon the physiological state of the animal. In a series of 63 separate determinations in which undisturbed mice were removed from a cage and an eosinophil count performed within 10 minutes, we found the average count to be 409 cells per cubic millimeter. The range of variation was from 70 to 1280 cells.

However, when more than one determination was made on the same animal the number of eosinophils was found to change in a consistent manner. This is shown by the first experiment, the data of which are tabulated in Table A. Eight undisturbed mice were removed from a cage and 5 determinations of the number of circulating eosinophils were made over a period of 4 hours. The first count (called the zero hour count) was made within 10 minutes after the animals were removed from their cage. The technique for obtaining blood was followed exactly as described for the direct method. Successive determinations of the eosinophils were made after an interval of 30 minutes, 1 hour, 2 hours and 4 hours. The actual number of eosinophils per cubic millimeter is shown in the first 5 columns and the percent change shown in the last 4 columns of Table A. It can be seen that during the first half hour there is no consistent change. In half the animals the number of eosinophils decreased slightly and in the remaining half they increased over the initial count.

TABLE A. SUCCESSIVE DETERMINATIONS OF CIRCULATING EOSINOPHILS IN NORMAL MICE

Animal	Stimulus	Eosinophil count					Per cent change*			
		0 Hr.	½ Hr.	1 Hr.	2 Hr.	4 Hr.	½ Hr.	1 Hr.	2 Hr.	4 Hr.
Normal mice	Handling and loss of blood	750	663	463	94	6	- 12%	- 38%	- 37%	- 99%
		200	225	150	38	0	+ 13%	- 25%	- 81%	- 100%
		206	174	94	25	6	- 15%	- 54%	- 88%	- 97%
		331	344	188	75	0	+ 4%	- 43%	- 77%	- 100%
		203	228	97	31	3	+ 12%	- 52%	- 85%	- 99%
		147	353	384	60	3	+ 140%	+ 160%	- 59%	- 98%
		209	228	122	41	—	+ 9%	- 42%	- 80%	—
		284	419	191	44	6	+ 48%	- 33%	- 85%	- 98%
		Average					+ 24%	- 16%	- 80%	- 99%

* Per cent change is computed as follows:

$$\left(\frac{\text{Number of cells at each successive count}}{\text{Number of cells at zero hours}} - 1 \right) \times 100.$$

In the hour determination however; all but one of the mice showed a decrease in the number of circulating eosinophils. After 2 hours all the animals showed an average decrease of 80% of the initial count; after 4 hours there was a 99% decrease. Thus within a period of 4 hours the eosinophils had almost completely disappeared from the tail blood.

There are at least two possible explanations for this change in the number of eosinophils. First, a local inflammatory reaction due to successive cuts on their tails. Second, the excitement due to handling the mice might have set up an "alarm reaction" which in some way affected the eosinophil cells.

A second experiment was designed to determine whether mild

stresses, not associated with trauma and loss of blood, would cause a decrease in eosinophils. A cage containing normal mice was opened and the mice shaken out of their bedding. The animals ran around the cage in mild excitement for a few minutes and then quieted down. Four hours later a blood count was made, and the following morning a second count was taken. It can be assumed that the 18 hour count, made after the mice had been left undisturbed overnight, would be approximately the same as the count would have been, if one had been made at 0 hours. The basis for this assumption is discussed later in this paper.

It can be seen from Table B that the eosinophil count made 4 hours after mild excitement was 90% lower than a similar count made after the same mice had been left undisturbed for 14 hours. This experiment indicates that the loss of blood or local inflammation of the tail is not the major factor causing the decrease in circulating eosinophils.

TABLE B. EFFECT OF MILD STIMULATION ON THE EOSINOPHILS OF NORMAL MICE

Stimulus	Eosinophil count			%* Change
	0 Hr.	4 Hr.	18 Hr.	
Cage cleaned. Mice shaken out of cotton	—	38	374	-90%
	—	34	544	-94%
	—	228	881	-74%
	—	9	331	-97%
	—	31	303	-90%
	—	122	859	-86%
	—	6	284	-98%
Average				-90%

* Per cent change: $\left(\frac{4 \text{ hour count}}{18 \text{ hour count}} - 1 \right) \times 100.$

A third experiment was designed to study in more detail the actual change in the eosinophil count after a single period of excitement or stress. In this experiment 7 groups of normal mice were used, 8 animals in each group. Two determinations were made of the number of circulating eosinophils in each animal. Between determinations the animals were returned to their cages for specific periods of time. The handling of the mice at the time of the first bleeding is considered a mild stress and is responsible for bringing about changes seen in the second count. See Table C. The first group had the two counts made approximately $\frac{1}{2}$ hour apart. During this time there was no significant change in the number of eosinophils. In the second group, in which the eosinophil determinations were taken one hour apart, all the animals showed a significant decrease, averaging approximately 40%. In the 2 hour group there was a decrease of 49%, and in the 4 hour group the decrease averaged 83%.

TABLE C. PROGRESSIVE CHANGE IN THE NUMBERS OF CIRCULATING EOSINOPHILS
IN NORMAL MICE FOLLOWING A MILD STRESS*

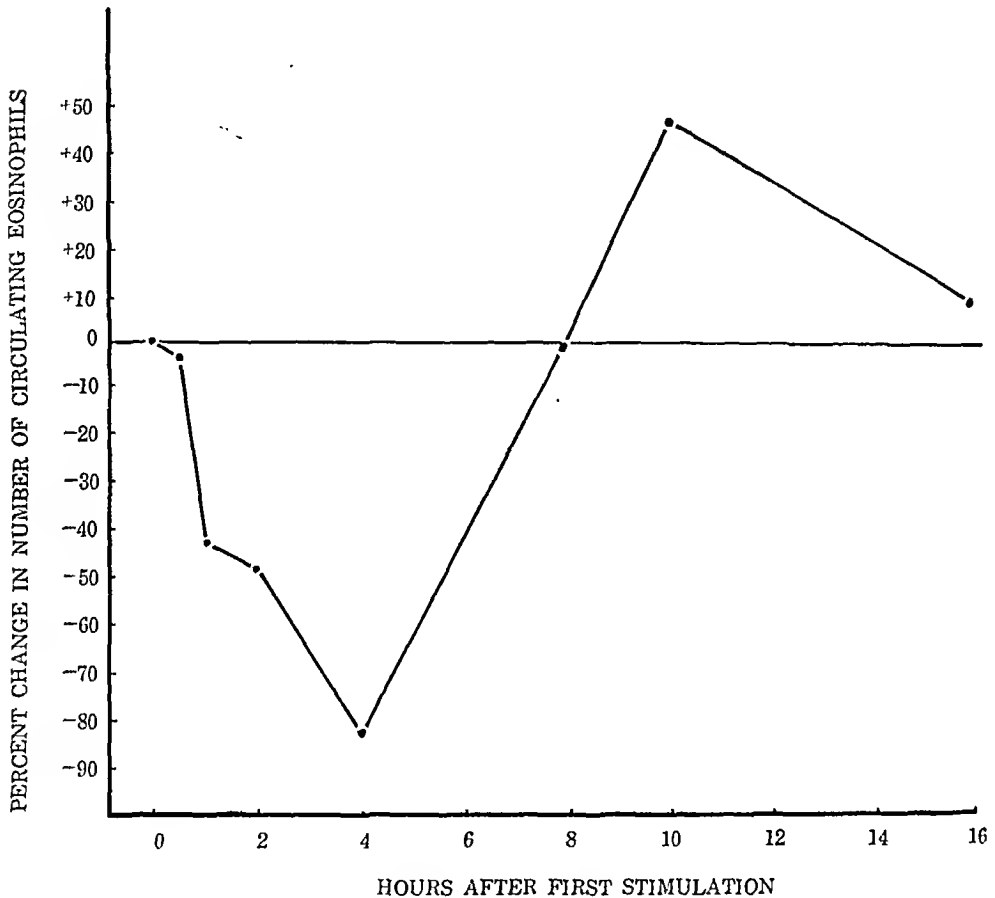
Group I			Group II			Group III			Group IV			Group V			Group VI			Group VII		
0	†	%†	0	1 Hr.	%	0	2 Hr.	%	0	4 Hr.	%	0	8 Hr.	%	0	10 Hr.	%	0	16 Hr.	%
569	463	-17%	206	63	-69%	375	213	-43%	125	38	-70%	206	225	+9%	85	107	+26%	325	403	+24%
413	428	+4%	881	438	-50%	303	125	-58%	219	36	-84%	88	153	+73%	275	394	+43%	284	194	-32%
806	780	-3%	1281	984	-23%	578	268	-54%	144	13	-91%	94	163	+74%	163	416	+155%	88	116	+32%
141	122	-13%	622	528	-15%	278	141	-49%	356	13	-96%	188	216	+15%	225	258	+15%	413	141	-66%
409	444	+9%	760	463	-38%	331	153	-54%	213	88	-59%	403	184	-54%	101	381	+100%	150	213	+42%
760	663	-11%	200	150	-25%	381	225	-41%	675	31	-95%	408	82	-80%	141	156	+11%	180	188	+4%
200	225	+13%	206	04	-54%	544	278	-49%	625	131	-79%	116	76	-34%	338	384	+14%	78	128	+68%
206	175	-15%	331	188	-43%	1081	647	-40%	400	38	-90%	141	128	-9%	703	768	+9%	107	94	-12%
Average		-5%	Average		-40%	Average		-49%	Average		-83%	Average		-1%	Average		+47%	Average		+9%

* These mice were removed from their cages, warmed, and a sample of blood taken from their tails. No other stimulus was applied. The mice were then put back in their cages for specific periods of time, after which a second sample was taken.

† Per cent change: $\left(\frac{\text{Number of cells at each successive count}}{\text{Number of cells at zero hours}} - 1 \right) \times 100$

In the 5th group, in which the second eosinophil determination was made 8 hours after the first, half of the animals showed an increase over the original zero hour count. In the 10 hour group all the animals showed an increase averaging 47%. Finally, in the last group there was a large variation, but the average of the 8 animals at 16 hours was approximately the same as the determination made at zero hours.

The changes shown in Table C are plotted in Figure 1.



CHANGE IN NUMBER OF CIRCULATING EOSINOPHIL CELLS IN NORMAL MICE FOLLOWING MILD STRESS.

Fig. 1

In summary, it can be emphasized that a mild stress produces a great decrease in the number of circulating eosinophils which lasts over a period of approximately 8 hours. Following this there is an increase in the number of circulating eosinophils and a return to normal by approximately 16 hours after the initial stimulation.

It would be expected, that if more than one mild stimulation were given, the effect would be cumulative. To test this hypothesis mice which had had two determinations made within $\frac{1}{2}$ hour were

checked again at 8 hours. In these animals the number of eosinophils still showed a decrease of 96%, whereas the average from a similar group of animals which had had only one stimulation returned to approximately the original level by 8 hours. By 20 hours the number of eosinophils was approximately normal again in the group with the double stimulation.

TABLE D. EFFECT OF DOUBLE STIMULATION ON THE EOSINOPHILS OF NORMAL MICE

Stimulus	Eosinophil count				Per cent change*		
	0 Hr.	$\frac{1}{2}$ Hr.	8 Hr.	20 Hr.	$\frac{1}{2}$ Hr.	8 Hr.	20 Hr.
Handling and loss of blood	556	463	3	278	-17%	-99%	-50%
	1275	1300	16	1077	+2%	-99%	-15%
	1047	1263	19	1472	+21%	-98%	+41%
	413	428	59	381	+3%	-86%	-8%
	806	750	3	681	-7%	-99%	-16%
	141	122	0	206	-13%	-100%	+46%
	409	444	19	578	+9%	-95%	+41%
Average					0%	-96%	+6%

* Per cent change:

$$\left(\frac{\text{Number of cells at each successive count}}{\text{Number of cells at zero hours.}} - 1 \right) \times 100.$$

TABLE E. EFFECT OF EPINEPHRINE INJECTIONS ON THE EOSINOPHILS OF NORMAL ANIMALS

Animal	Stimulus	Eosinophil count		Per cent change*
		0 Hr.	4 Hr.	
Intact mice	5 gamma Epinephrine	763	38	— 95%
		238	6	— 97%
		750	6	— 99%
		200	0	—100%
		206	6	— 97%
		331	0	—100%
		1060	19	— 98%
Average				— 98%
Intact rats	200 gamma Epinephrine	369	34	— 91%
		209	103	— 51%
		188	16	— 91%
		363	59	— 84%
Average				— 79%

* Per cent change: $\left(\frac{\text{4 hour count}}{\text{0 hour count}} - 1 \right) \times 100.$

If this decrease in eosinophils is part of the mechanism by which animals respond to stress, then injections of epinephrine should produce approximately the same effect. To determine whether this was true, 8 mice were injected with 5 gamma of epinephrine, and eosino-

phil determinations made before the injection and 4 hours later. The results obtained are shown in Table E. All 8 animals showed a decrease averaging 98%. Epinephrine injected into rats also produced approximately the same effect. In 4 animals the average decrease was 79% at 4 hours.

In 5 separate experiments described above, the numbers of eosinophils in mice and rats were found to vary in a consistent manner following stress. There was a decrease in the number of circulating eosinophils beginning within one hour after the stress was first applied, and lasting for 7 hours or more depending upon the degree and amount of stress. If the stress is sufficiently great there will be an almost complete disappearance of the eosinophils from the tail blood. Following this period of eosinopenia there was a second period in which an eosinophilia occurred. In most animals this disappeared by 16 hours.

These data indicate that a great deal of consideration should be given to the procedures used to obtain blood from normal mice and rats, if an accurate determination is to be made. The animals should not be handled or disturbed for at least 16 hours prior to a blood sampling. Once the mice are disturbed by removing them from their cages, etc., the blood sample should be taken as soon as possible.

The authors would like to point out that the eosinophil picture following mild stress is characteristic for the C 57 Brown mice used, and might be quite different in other strains of mice.

B. Eosinophil determinations in adrenalectomized mice

In a series of 454 determinations of the level of circulating eosinophils in adrenalectomized mice, the average number of cells was found to be 349 per cubic millimeter. There was a wide variation of from 10 to 1653 eosinophils. Thus the average count and the range of variability in the adrenalectomized mouse was not significantly different from that of the intact animal.

One of the most important factors to consider in these animals is

TABLE F. EFFECT OF MILD STIMULATION ON THE EOSINOPHILS
OF ADRENALECTOMIZED MICE

Animal	Stimulus	Eosinophil count				Per cent change*		
		0 Hr.	2 Hr.	4 Hr.	8 Hr.	2 Hr.	4 Hr.	8 Hr.
Adrenalectomized mice	Handling and loss of blood	134	131	138	144	- 2%	+ 3%	+ 7%
		331	297	347	306	-10%	+ 5%	- 8%
		76	138	225	228	+82%	+196%	+200%
		219	213	353	441	- 3%	+ 61%	+101%
		406	469	600	638	+16%	+ 48%	+ 57%
		234	266	316	209	+14%	+ 35%	- 11%
Average					+16%	+ 58%	+ 59%	

* Per cent Change:

$$\left(\frac{\text{Number of cells at each successive count}}{\text{Number of cells at zero hours}} - 1 \right) \times 100.$$

the change in the number of circulating eosinophils after various stress stimuli. In the first experiment 6 mice were adrenalectomized and maintained by the addition of 1% salt to their drinking water. Three days later, 4 successive blood determinations were made over a period of 8 hours. The data obtained are shown in Table F. It will be noted that the number of eosinophils in the circulation of the mouse's tail actually increased in the succeeding determinations. In all these animals the removal of the adrenal prevented the eosinopenia which occurs in normal animals following stress.

TABLE G. EFFECT OF EPINEPHRINE INJECTIONS ON THE EOSINOPHILS OF ADRENALECTOMIZED MICE

Animal	Stimulus	Eosinophil count		Per cent change*
		0 Hr.	4 Hr.	
Completely adrenalectomized mice	5 gamma Epinephrine	181	263	+45%
		144	250	+74%
		244	263	+ 8%
		313	488	+56%
		363	438	+21%
		494	519	+ 5%
		188	300	+60%
		294	513	+74%
		Average		+43%

$$* \text{ Per cent change: } \left(\frac{4 \text{ hour count}}{0 \text{ hour count}} - 1 \right) \times 100.$$

If 5 gamma of epinephrine is injected into each of the adrenalectomized mice, the number of eosinophils also increases over a 4 hour period. These data are shown in Table G. The same general increase (52%) also occurred in 4 adrenalectomized rats following 200 gamma of epinephrine, subcutaneously. Thus stress and epinephrine injections do not cause a decrease in the number of circulating eosinophils in adrenalectomized mice and rats.

In the next experiment the effects of an extract of the adrenal cortex was tested in adrenalectomized mice. Five mice received an injection of 0.05 cc. Lipo-Adrenal Cortex (Upjohn Company) and eosinophil determinations made over a period of 12 hours. The film method described earlier was used to determine the number of eosinophils.

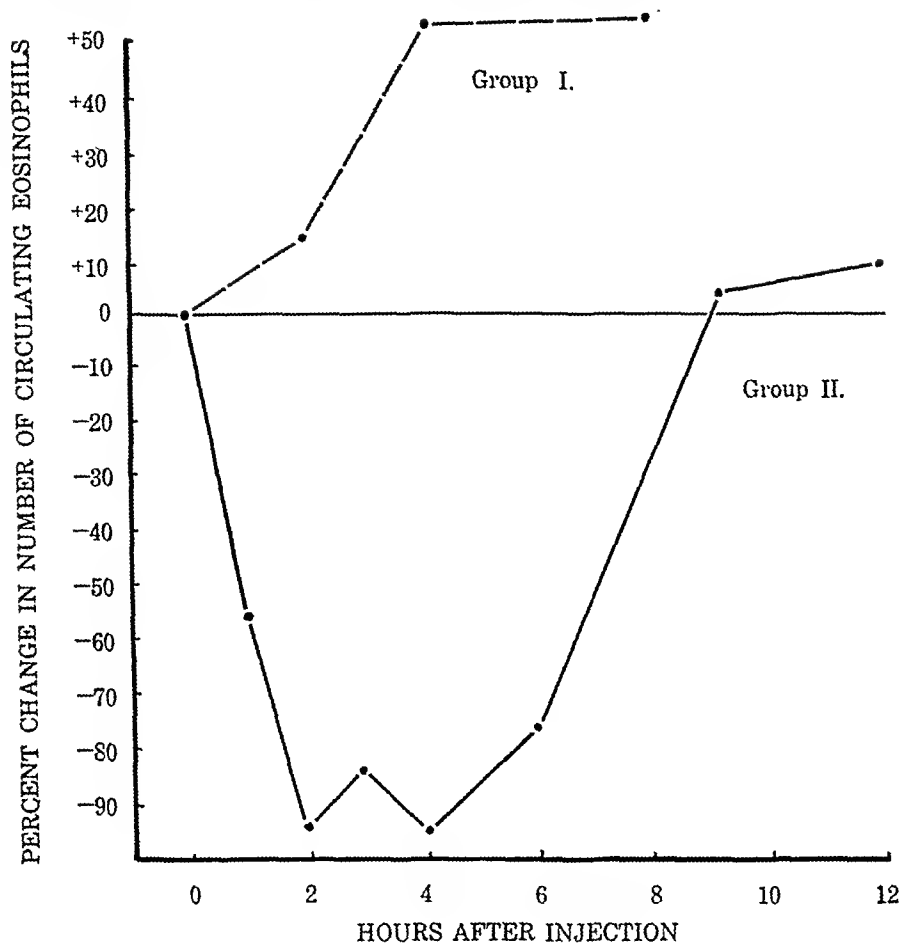
The data shown in Table H and Figure 2 show that the cortin produces a marked decrease in the eosinophil count beginning within the first hour, and lasting for more than 6 hours. By 9 and 12 hours the number of circulating eosinophils has returned to normal. Thus in completely adrenalectomized animals eosinopenia can be obtained by injecting cortical extracts. The time of response and type of curve obtained is almost identical to that produced in the normal animal after stress.

However, if mice are subjected to a mock operation, unilateral

TABLE H. EFFECT OF ONE INJECTION OF CORTIN ON THE EOSINOPHILS OF ADRENALECTOMIZED MICE

Injection	Eosinophil count								Per cent change*						
	0	1 Hr.	2 Hr.	3 Hr.	4 Hr.	6 Hr.	9 Hr.	12 Hr.	1 Hr.	2 Hr.	3 Hr.	4 Hr.	6 Hr.	9 Hr.	12 Hr.
0.05 cc. Cortin	400		36		26	0	807	453		- 91%		- 93%	-100%	+102%	+13%
	638		61		53	226	126	518		- 90%		- 92%	- 58%	- 80%	-19%
	647		0		0	82	306	291		-100%		-100%	- 87%	- 53%	-55%
	445	173		51		134	514	632	-61%		-89%		- 70%	+ 16%	+42%
	443	225		113		15	593	750	-49%		-74%		- 97%	+ 34%	+69%
Average									-55%	- 94%	-82%	- 95%	- 82%	+ 4%	+10%

* Per cent change: $\left(\frac{\text{Number of cells at each successive count} - 1}{\text{Number of cells at zero hours}} \right) \times 100.$



CHANGE IN NUMBER OF CIRCULATING EOSINOPHIL CELLS IN ADRENALECTOMIZED MICE.

Group I. Following mild stress.

Group II. Following a subcutaneous injection of 0.05 cc Cortin (Lipo-Adrenal Cortex)

FIG. 2

adrenalectomy, or if remnants of one or both adrenals are left within the body cavity at the normal site or are transplanted to the spleen or kidney, then a decrease in eosinophils will follow the epinephrine injections. This is shown in Table I. It will be noted that in many of these animals in which there has been a regeneration of the adrenals, a typical eosinopenia does not occur. There is an average decrease of 65%, and many animals show only a 30 or 40% decrease, as opposed

TABLE I. EFFECT OF EPINEPHRINE INJECTIONS ON THE EOSINOPHILS OF MICE WITH PARTIAL REGENERATION OF ONE OR BOTH ADRENALS

Animal	Stimulus	Eosinophil count		Per cent change*
		0 Hr.	4 Hr.	
Adrenalectomized mice in which there has been partial regeneration of one or both adrenals	5 gamma Epinephrine	769	75	-90%
		119	31	-74%
		138	94	-32%
		407	250	-39%
		150	9	-94%
		241	110	-54%
		275	169	-39%
		450	113	-75%
		Average		

$$* \text{ Per cent change: } \left(\frac{4 \text{ hour count}}{0 \text{ hour count}} - 1 \right) \times 100.$$

to a 98% decrease in normal animals (Table E). The tentative conclusion drawn from this is that the regenerated adrenal is not as efficient as normal adrenals. Furthermore, the data indicate that the pattern of eosinophil change following epinephrine can be used to determine whether an animal is completely adrenalectomized.

To test this last suggestion an experiment was set up to determine whether there was any correlation between the response of the eosinophils to epinephrine and the ability of the mice to live after removal of salt from their drinking water. Thirty-six animals were adrenalectomized and maintained for at least 30 days on a 1% saline solution. Five gamma of epinephrine was injected and an eosinophil count taken before injection and 4 hours later. On the basis of their response the mice could be arranged into 3 groups: Group I. Either no change or an actual increase in number of eosinophils during the 4 hours after epinephrine. Group II. Slight decrease of from -1 to -30%. Group III. A greater decrease of from -31 to -100%.

TABLE J. CORRELATION OF THE EOSINOPHIL RESPONSE OF ADRENALECTOMIZED MICE TO EPINEPHRINE AND THE ABILITY OF THESE MICE TO LIVE AFTER REMOVAL OF SALT FROM THEIR DRINKING WATER

Group	Per cent change* in eosinophils after epinephrine	Total No. animals	Number dead after removing salt			Total dead	Total alive
			0-10 day	10-20 day	20-30 day		
I	0 to + 92%	15	12	2	0	14	1
II	- 1% to - 30%	4	2	0	1	3	1
III	-31% to -100%	17	0	0	1†	1	16

$$* \text{ Per cent change is computed as follows: } \left(\frac{4 \text{ hour count}}{0 \text{ hour count}} - 1 \right) \times 100.$$

† This animal at autopsy had focal areas of purulent material throughout the liver. Death probably caused by infection.

The mice were then placed in a large cage and fed a normal diet without additional saline. Daily checks were made of the cage and all deaths noted. The results are tabulated in Table J.

In Group I, 14 of the 15 mice died within 20 days. The remaining mouse was reinjected with epinephrine and this time a decrease occurred indicating that the adrenal was functioning. In Group II, 3 of the 4 animals died within 30 days. In Group III, only one animal died, apparently from an infection rather than adrenal insufficiency.

In summary, it is concluded that animals showing marked decrease in eosinophils after epinephrine injections had sufficient regeneration of the adrenal cortex to maintain them without additional saline in their drinking water. Mice which showed only a slight decrease, or an increase in eosinophils after epinephrine did not have sufficient regeneration of the cortex to maintain themselves when the saline was removed.

The authors have supplemented this experiment by autopsying many other mice. Animals which responded to epinephrine were immediately autopsied and in many cases small remnants of the adrenal tissue were seen. In a few cases, however, no actual adrenal tissue could be found macroscopically. In these animals it was assumed that adrenal rests probably had occurred in some of the organs, such as the gonads. In no case has adrenal tissue been found in animals which did not respond to epinephrine.

C. Eosinophil determinations in splenectomized mice

In a series of 13 separate determinations the number of circulating eosinophils in undisturbed splenectomized mice was found to average approximately 577 cells. The range of variability was from 169 to 1653 cells. This is not considered a significant increase over the count obtained for normal animals. However, the mice had only been splenectomized for a period of two weeks.

In the first experiment successive blood counts were made over a period of 4 hours on 5 splenectomized mice. The data obtained are shown in Table K. In these animals a rapid decrease in the number of circulating eosinophils occurred which was similar to the response of intact animals shown in Table A. This indicates the spleen is not involved in the rapid decrease in circulating eosinophils following mild stress.

In a second experiment 6 mice were adrenalectomized and splenectomized in a one stage operation. In these animals the average number of eosinophils increased after mild stress as shown in Table K. This response was similar to that obtained from adrenalectomized mice (Table F). These data suggest that the spleen is not involved in the increase in numbers of eosinophils which occurs in adrenalectomized animals.

TABLE K. EFFECT OF MILD STRESS ON THE EOSINOPHILS OF SPLENECTOMIZED AND ADRENALECTOMIZED-SPLENECTOMIZED MICE

Animal	Stimulus	Eosinophil count					Per cent change*			
		0 Hr.	1 Hr.	2 Hr.	4 Hr.	8 Hr.	1 Hr.	2 Hr.	4 Hr.	8 Hr.
Splenectomized mice	Handling and blood loss	709	472	163	128		-33%	-70%	-82%	
		1653	472	88	91		-71%	-95%	-94%	
		906	506	85	16		-44%	-91%	-98%	
		169	150	69	3		-11%	-59%	-98%	
		500	544	200	6		+9%	-60%	-99%	
		Average								
Adrenalectomized-splenectomized mice.	Handling and blood loss	331		428	481	547		+29%	+45%	65%
		563		563	969	753		0	+72%	+34%
		1038		1203	1131	1106		+16%	+9%	7%
		178		341	347	406		+92%	+95%	+128%
		979		1266	1141	1406		+29%	+17%	+44%
		288		269	281	356		-7%	-2%	+24%
		Average								
							+27%	+39%	+50%	

* Per cent change: $\left(\frac{\text{Number of cells at each successive count}}{\text{Number of cells at zero hours}} - 1 \right) \times 100.$

D. Eosinophil determination in hypophysectomized mice

In a series of 41 different determinations made in undisturbed hypophysectomized mice, we found the average eosinophil count to be 290 cells per cubic millimeter. The range of variation was from 28 to 866 cells. This count is not considered significantly different from that of normal mice.

The effects of handling, epinephrine, and ACTH injections are shown in Table L. It can be seen readily that handling and epinephrine injections caused a decrease in the number of circulating eosinophil cells during a 4 hour period. This decrease occurred in all animals during a period of from 3 to 14 days after hypophysectomy. Injections of 50 gamma of ACTH produced a much greater decrease, averaging 87%, during the same period of time.

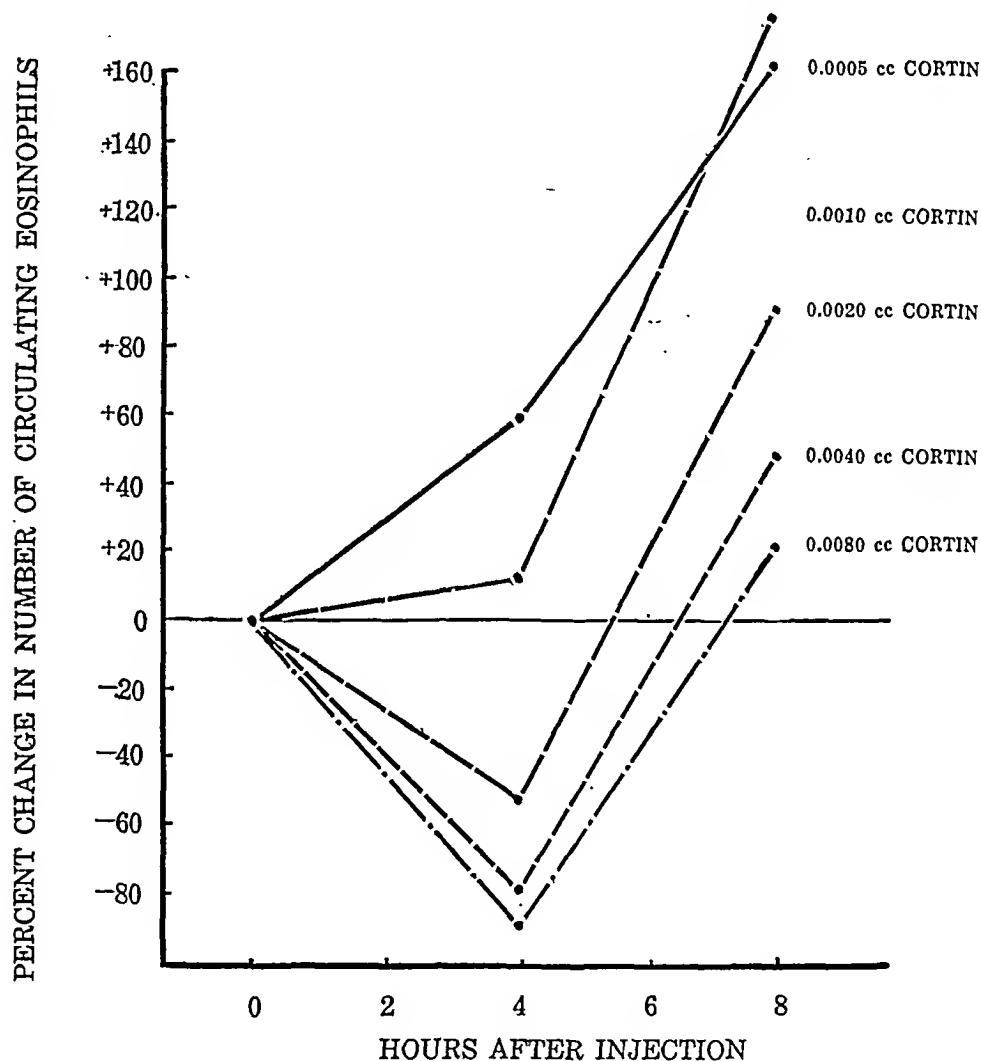
TABLE L. EFFECTS OF VARIOUS STIMULI ON THE EOSINOPHILS OF HYPOPHYSECTOMIZED MICE

Stimulus	Days A/P	Eosinophil count				Per cent change		
		0 Hr.	2 Hr.	4 Hr.	8 Hr.	2 Hr.	4 Hr.	8 Hr.
Handling and blood loss	3	275		222	381		- 19%	+ 39%
	3	491		453	488		- 8%	- 1%
	3	613		488	541		- 20%	- 12%
	3	603		344	353		- 43%	- 41%
	3	250		147	181		- 41%	- 28%
	7	147		82	141		- 44%	- 4%
	7	166		44	297		- 73%	+ 79%
	7	303		188	328		- 38%	+ 8%
	7	28		3	31		- 89%	+ 11%
	Average						- 42%	+ 6%
5 gamma Epinephrine	7	107		56			- 48%	
	7	103		56	241		- 46%	+ 134%
	7	241		94	275		- 61%	+ 14%
	7	266		110	328		- 62%	+ 23%
	7	172		110	110		- 36%	- 36%
	7	209		144	247		- 31%	+ 18%
	7	144		110	138		- 24%	- 4%
	Average						- 44%	+ 25%
5 gamma Epinephrine	14	113		38	69		- 66%	- 39%
	14	328		250	291		- 24%	- 11%
	14	403		313	391		- 22%	- 3%
	14	197		9	85		- 95%	- 57%
	14	313		141	—		- 55%	—
	14	534		294	394		- 45%	- 26%
	14	191		44	216		- 72%	+ 13%
	Average						- 54%	- 21%
50 gamma ACTH	4	147	41	53	191	-72%	- 74%	
	4	219	50	31		-77%	- 86%	+ 30%
	4	134	28	19		-79%	- 86%	
	4	172	16	0		-91%	-100%	
	4	82	3	13		-96%	- 84%	
	4	266	85	13		-69%	- 95%	
	4	234	113	44		-52%	- 81%	
	Average					-77%	- 87%	+ 30%

E. Effect of various steroid materials on the eosinophil count of adrenalectomized mice and rats

It has already been shown in section B, Table H, that extracts of the adrenal cortex will produce temporary eosinopenia in adrenalectomized mice. It is of importance to determine whether this eosinopenia is a specific quantitative response.

The first experiment was designed to determine whether varying doses of cortin would produce different levels of response in adrenalectomized mice. The cortin was diluted with corn oil so that each animal received 0.03 cc. injection of oil subcutaneously in the dorsal region



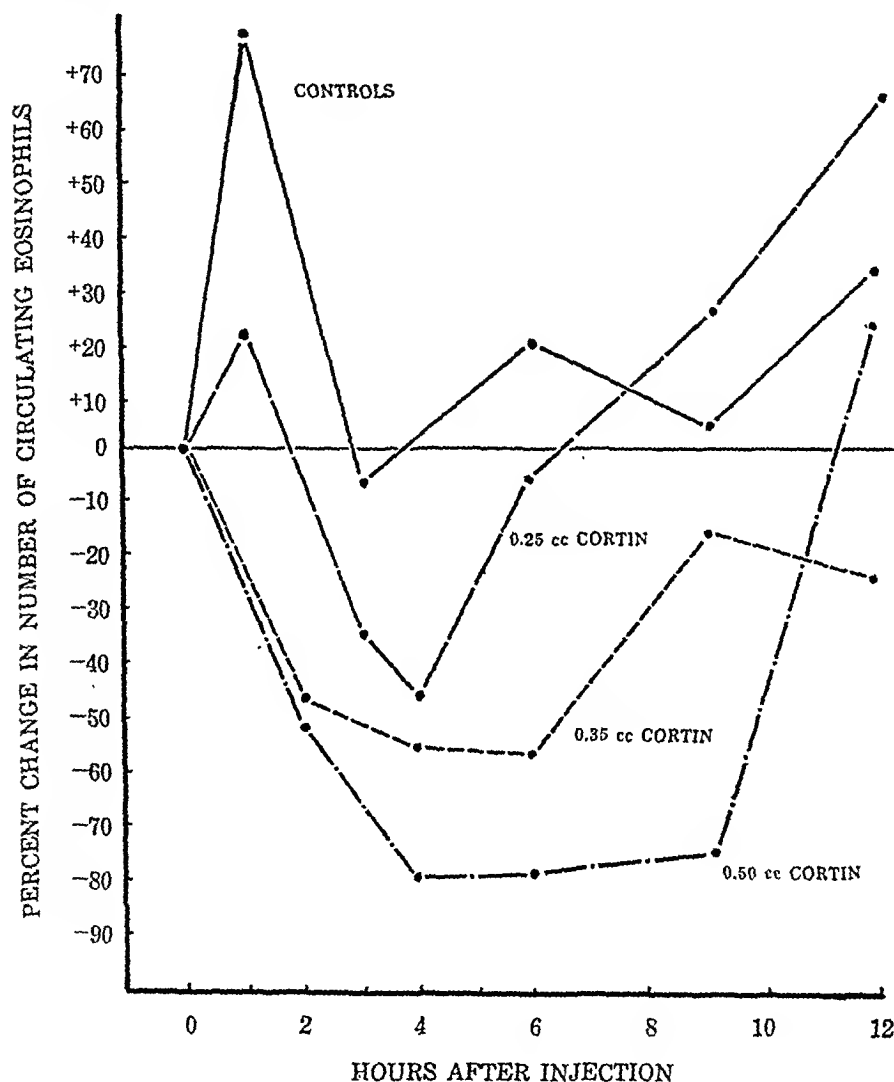
CHANGE IN NUMBER OF CIRCULATING EOSINOPHIL CELLS FOLLOWING A SUBCUTANEOUS INJECTION OF VARYING DOSES OF CORTIN IN ADRENALECTOMIZED MICE.

FIG. 3

of the back. Eosinophil determinations were made before the injections and at 4 and 8 hours afterwards. The data from 29 mice were calculated and plotted in Figure 3. The smallest dose, equal to 0.0005 cc. of the original extract, did not produce an eosinopenia, but caused instead an average increase of 59% and 162% at 4 and 8 hours respectively. The next dose produced a decrease in 3 out of the 6 ani-

mals and the average was 8% increase at 4 hours. The third and all larger doses produced a marked decrease in all of the animals at the 4 hour determination. At the 8 hour determination the averages of all groups were higher than the initial zero hour count. It can be clearly seen in Figure 3 that the extent of the decrease in eosinophils was correlated with the amount of cortin injected.

The same type of response occurred in rats, except that the amount of cortin needed to produce the change was approximately



CHANGE IN NUMBER OF CIRCULATING EOSINOPHIL CELLS FOLLOWING A SUBCUTANEOUS INJECTION OF VARYING DOSES OF CORTIN IN ADRENALECTOMIZED RATS.

FIG. 4

100 times greater than that used in the mouse. See Figure 4. It can be noted that as the dose of cortin is increased, the per cent decrease in the circulating eosinophils becomes much greater, and the period of eosinopenia also becomes longer. These data are based upon averages obtained from 24 rats.

It is apparent from the data just described that mice are much

TABLE M. EFFECT OF VARIOUS STEROID MATERIALS ON THE EOSINOPHILS OF ADRENALECTOMIZED MICE

Material injected	Dose	Eosinophil count			Per cent change*	
		0 hr.	4 hr.	8 hr.	4 hr.	8 hr.
Theelin	33 γ	125	119	238	- 5%	+ 90%
	33 γ	344	238	331	- 31%	- 4%
	33 γ	350	681	606	+ 95%	+ 73%
	33 γ	206	369	389	+ 78%	+ 89%
	Average				+ 35%	+ 62%
Testosterone Propionate	750 γ	238	555	252	+133%	+ 6%
	750 γ	467	421	614	- 10%	+ 31%
	750 γ	197	107	260	- 46%	+ 32%
	Average				+ 26%	+ 23%
Desoxycorticosterone	150 γ	244	88	106	- 64%	- 57%
	150 γ	413	94	319	- 72%	- 23%
	150 γ	206	75	256	- 64%	+ 24%
	Average				- 67%	- 19%
	25 γ	481	581	363	+ 21%	- 25%
	25 γ	250	213	281	- 15%	+ 12%
	Average				+ 3%	- 7%
	3 γ	250	6	206	- 98%	- 18%
	3 γ	150	0	162	-100%	+ 8%
	3 γ	125	12	218	- 90%	+ 74%
Compound E	3 γ	350	19	681	- 95%	+ 95%
	Average				- 96%	+ 40%
Compound A	25 γ	116	31	237	- 74%	+103%
	25 γ	338	31	356	- 91%	+ 5%
	25 γ	263	56	406	- 79%	+ 54%
	Average				- 81%	+ 54%

* Per cent change: $\left(\frac{4 \text{ or } 8 \text{ hour count}}{0 \text{ hour count}} - 1 \right) \times 100.$

more sensitive to cortin than rats. In both animals the amount of decrease in the circulating eosinophils is correlated with the dose of cortin given. A brief period of eosinophilia usually follows the eosinopenia.

Various other steroids and oils have been injected to determine their effect on the circulating eosinophils. The data obtained after

using a few of these materials are shown in Table M. The eosinophil counts were made by the film procedure described earlier. Theelin (Keto-hydroxy estratriene) and testosterone propionate have no effect on the circulating eosinophil numbers. A relatively large dose of desoxycorticosterone produced an average decrease of 67%, whereas smaller doses had no effect. The eosinophils seem to be extremely sensitive to oxycorticosteroids such as Compound E (11 dehydro-17-hydroxy-corticosterone). As little as 3 gamma of the Compound E dissolved in acetone and corn oil produced a maximum eosinopenia (96% decrease) in 4 hours. In other experiments to be published later, less than one gamma was found to be effective in reducing the eosinophil count.

Compound A (Dehydro-corticosterone) decreased the numbers of eosinophils, but was not nearly so effective as Compound E.

DISCUSSION

This paper has presented two techniques for the determination of circulating eosinophils in the mouse and rat. The direct procedure is based upon the principle that the eosinophil is the most resistant cell of the blood. A 15% acetone solution used in a 1:20 dilution with blood gave the highest and most consistent eosinophil count. In this mixture of blood and diluent, all the red blood cells and the majority of white blood cells except the eosinophil cells were either made indistinct or destroyed. The second procedure used was similar to techniques used in routine clinical and animal hematology. The only modification was in an improved staining procedure. Wright's and Giemsa's Blood Stains were used together in a coplin jar to produce a better staining of the eosinophil granules in the mouse. Total leukocyte counts were determined in a 4% acetic acid solution, and differential counts were made from stained blood films.

In general the film procedure is used to determine over-all blood changes following the injection of material having unknown physiological action. The direct method is quicker, easier, and more reliable and is used for the detection of quantitative variations of the eosinophils following different doses of the adrenal cortical hormone, etc.

An estimate of the over-all error of the direct technique was obtained by making counts of successive drops of blood taken directly from a mouse's tail. The standard error was found to be 1.7 times the square root of the number of cells counted. This includes the variation in eosinophils in successive drops of blood obtained from the tail as well as errors in technique, instruments, etc.

The results obtained in this paper clearly indicate that there is no significant difference in the initial eosinophil counts of normal, adrenalectomized, or hypophysectomized mice, just after removal from their cages. However, succeeding blood determinations following mild stress or epinephrine injections showed a marked difference

between the three groups of animals. In normal intact mice there is a marked eosinopenia lasting for approximately 7 hours, followed by a slight eosinophilia. See Figure 1. In the adrenalectomized mice a slight eosinophilia occurs. Eosinopenia does not occur except after injections of adrenal cortical extracts. See Figure 2. In the hypophysectomized mice a decrease in the number of eosinophils occurred, but it was not so marked as in the normal animals. However, a complete eosinopenia was obtained by injections of ACTH.

These data can best be interpreted as follows: Stress or epinephrine injections produce a release of oxycorticosteroids from the adrenal cortex which causes a decrease in the number of circulating eosinophils. The spleen is not involved in these reactions.

In a recent review, Long (1947) pointed out that there are three possible paths through which the epinephrine may act to cause a release of cortin:

1. Directly on the pituitary gland causing a release of ACTH which acts upon the adrenal cortex.
2. By producing a drop in the level of cortical hormone in the blood, which in turn produces an increased release of ACTH by the pituitary.
3. Directly on the adrenal cortex causing a release of the cortical hormone.

The data presented in this paper indicate that there may be a combination of two or more of these paths in the mouse. As already indicated, epinephrine produces a marked decrease in circulating eosinophils if both the pituitary and adrenal are present. If the adrenal cortex is removed, epinephrine injections will not produce the decrease. If the pituitary is removed, epinephrine produces a decrease in the eosinophils, but a complete eosinopenia does not occur. This indicates that in hypophysectomized animals the epinephrine acts directly on the adrenal cortex to produce a release of cortical hormone. The amount of hormone released is small in comparison with the amount released after ACTH is injected.

These observations agree with published results of Vogt (1944). She was able to obtain increased cortical hormone output in a dog following perfusion of the adrenal with epinephrine. The effect was not mediated through the pituitary. However, Long and Fry (1945) noted that there was no measurable decrease in cholesterol or ascorbic acid in the adrenals of hypophysectomized rats following epinephrine injections. It is possible that the amount of hormone released is too small to be detected by their methods.

There is a second possible interpretation of these results. Experiments of Dean and Greep (1948) have suggested that release of desoxycorticosteroids is not under pituitary control in the rat. If the epinephrine produced a release of large amounts of this material, then it is conceivable that a decrease of the eosinophils may follow.

The use of epinephrine injections to test the completeness of adrenalectomy should prove to be very useful. It can be used to screen a group of animals to determine which ones have tissue capable of producing cortical hormones. This is particularly important in various assay procedures. It is now possible to eliminate all incompletely adrenalectomized animals before an experiment is set up on the basis of their eosinophil response after epinephrine. This is much quicker and easier than the transplantation procedure used by Kroc (1942). Furthermore, the relation of the hormone secretion and regeneration can now be followed. For example, it has been observed that in a group of animals very few will show a positive test for functional adrenal tissue during the first few days after adrenalectomy. However, after that, a slight eosinopenia begins to occur in some after epinephrine.

This response becomes progressively greater during the following weeks, indicating that an animal should be tested for adrenal cortical tissue each time it is to be used in an assay procedure.

In many of the animals which have regenerated adrenal tissue a complete eosinopenia is not obtained even after 3 months or more after adrenalectomy and regeneration. Following a 5 gamma injection of epinephrine the eosinophils do not decrease in the same proportion as in normal animals. Yet these animals survive after removal of salt from their drinking water, and under good conditions will grow and appear healthy. However, following a mild stress, these animals seem to die very easily. For example, after a series of blood determinations were made, many of the mice went into shock and died.

In a similar manner, animals which had adrenal cortical transplants in the spleen and kidney do not respond to adrenalin by complete eosinopenia (unpublished data). In 1940 Eversole, Edelmann and Gaunt pointed out that rats, with adrenals transplanted to various parts of the body, could survive removal of salt from their drinking water, but could not cope with intraperitoneal injections of water in the same manner as normal animals. This is in agreement with results obtained by many other authors, indicating that a regenerated cortex is functionally inadequate when the animal is subjected to intense or prolonged stress (Wyman and Tum Suden, 1937; Williams, 1947; Turner, 1948).

At the present time the authors are unable to explain the increase in the level of eosinophils in adrenalectomized animals. It has been noted in many mice and rats that an increase of as much as 200% is common following an initial eosinophil determination. This increase may be a local effect caused by cutting the tail of the animals, or possibly by a general hemoconcentration, or by stimulation of the bone marrow by epinephrine or some other compound. However, the spleen is not responsible for this increase, inasmuch as it occurs in splenectomized-adrenalectomized mice.

Graduated doses of an adrenal cortical extract produce different degrees of eosinopenia in adrenalectomized rats and mice. The eosinopenia is transitory, usually lasting less than 8 hours, and is followed by an eosinophilia. In all the materials used, this reaction was found to be specific for the adrenal cortical hormones, especially the oxycorticosteroids. The response was not obtained when sex hormones, oils, and various other materials were injected.

The factors presented in this paper form a background for a suggested technique for measuring exogenous adrenalcortical extracts, to be published in the near future.

SUMMARY

A direct procedure for determining eosinophils in mice and rats is presented along with a modified procedure for staining blood films in coplin jars.

A method of obtaining blood from mice is described in detail. This method was found to give reasonably consistent eosinophil counts when succeeding drops of blood were taken from the tail. The standard error of this procedure was found to be 1.7 times the square root of the number of eosinophils counted.

These techniques were applied to mice and rats and the following results obtained:

In normal mice, mild stress or epinephrine injections produced a great decrease in the number of circulating eosinophils. This eosinopenia lasted for approximately 7 hours and was followed by a period of eosinophilia. By increasing the amount of stress, a greater decrease in eosinophils occurred lasting over a longer period of time.

Removal of the adrenal cortex prevented the eosinopenia due to stress and epinephrine; instead a slight eosinophilia occurred after each determination. However, the eosinopenia was obtained after injections of adrenal cortical hormones. The degree of response was correlated with the amount of hormone injected. Sex hormones, oils, and low doses of desoxycorticosteroids did not produce an eosinopenia in adrenalectomized mice.

Evidence was presented which indicates that the change in the number of circulating eosinophils can be used to determine whether the animal has functional cortical tissue present. If a decrease of 30% or more in the number of circulating eosinophils occurs, then there is sufficient adrenal cortical tissue present to maintain the mouse after removal of the excess salt in its drinking water.

The spleen is not involved in the rapid decrease in the circulating eosinophils, nor in the eosinophilia of adrenalectomized mice.

In hypophysectomized mice, stress and epinephrine produced a decrease of from 40% to 50% in the number of circulating eosinophils. ACTH produced a greater effect, averaging an 87% decrease when 50 gamma was injected.

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EXCRETION OF GLUCOCORTICOIDS IN THE NEWBORN¹

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THE ADRENAL gland of the newborn infant differs greatly from that of the adult person. At birth the adrenals are relatively large structures and the cortex is composed of an outer or true cortical part and an inner part, the fetal cortex. This fetal cortex grows rapidly during the last trimester of fetal life and at birth or just previous to birth begins to degenerate. According to Benner (1940) this process is slow for the first 3 days of life but after that time proceeds rapidly for 2 to 3 weeks. This phenomenon was first observed by Scheel in 1908 and although numerous investigations have been carried out since that time, the function of the fetal cortex and the significance of its rapid involution are still not understood.

Grollman (1936) and Broster (1937) have suggested that this zone might be androgenic tissue, but attempts by Gersh and Grollman (1939) and by Carnes (1940) to demonstrate the presence of androgenic substance in the fetal adrenal have failed. In the following study an attempt has been made to determine whether the fetal cortex produces glucocorticoids, those adrenal hormones which promote the conversion of body protein to carbohydrate. As glucocorticoids are excreted in urine their measurement affords a relative means of evaluating the activity of the adrenal cortex with regard to this function.

CLINICAL MATERIAL AND METHODS

A total of twelve healthy full term and seven premature babies were studied at the Hospital of the University of Pennsylvania, Philadelphia. As only very small amounts of active adrenal substances are excreted by the newborn, in each case the urine had to be collected over a period of 3 to 4 days in order to obtain a satisfactory bioassay. In thirteen of these infants the urine was completed by the sixth day of life. In the others the collection was made at varying times between the ninth and thirty-seventh day of life. The prematurity of the babies varied from four to eight weeks and all infants

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weighed less than 2500 gms. and were considered to be well at the time of collection of the urine.

Two premature babies born of diabetic mothers and suffering from atelectasis were studied at the Royal Victoria Montreal Maternity Hospital. These cases are included through the courtesy of Dr. C. H. Read.

Baby M, a male, was delivered by Caesarian section about one month prior to term. At about 13 hours of age, he began to have moderately labored respirations. There was relative dullness over the left lung field with almost absent breath sounds. A clinical diagnosis of atelectasis was confirmed by radiological examination. The infant was put in an incubator and given continuous oxygen. The following day there was considerable improvement and on the fifth day of life the lungs were fully expanded. Urine collection began 4 hours after birth and was continued for seventy-two hours.

Baby B, a male was delivered by Caesarian section one month prior to term. The mother had been a severe diabetic for 10 years. At birth the baby was limp with a moderate amount of edema. He was placed in an incubator and given oxygen. About one half hour later, the baby became dyspneic and cyanosis was apparent if the oxygen was stopped. The clinical findings of complete collapse of the left lung with partial collapse of the right lung were confirmed by x-ray. There was slight improvement on the following day with some aeration of the right lung, and gradual improvement from then on, but the lungs were not clear clinically and radiologically until the seventh day of life. The first urine collection began at 6 hours after birth and was continued for seventy-two hours. Subsequent collections were made at the seventh, eleventh and fifty-fourth days of life.

On the possibility that the placenta might be storing glucocorticoids and allowing them to pass to the fetus, three placentae were examined separately for their content of glucocorticoids. The whole placenta was mixed in a Waring blender with $1\frac{1}{2}$ times its volume of water. The mixture was acidified with hydrochloric acid to pH₂ and from that point treated in a similar manner as the urine specimens. The weight of the placentae varied from 240 gm. to 500 gm.

The glucocorticoids were determined by the bioassay method of Venning, Kazmin and Bell (1946) which is dependent upon the glycogen deposited in the livers of fasted adrenalectomized mice. The results are expressed in terms of the standard 11-dehydro-17 hydroxy corticosterone or cortisone. Previously the term glycogenic units was used.

RESULTS

The results on the healthy babies are charted in Fig. 1. By pooling several 24-hour specimens it was possible, by the biological assay, to detect small amounts of adrenal corticoids in the urine of these infants. The glucocorticoids ranged from 4 to 17 $\mu\text{g}/24$ hours, the average excretion being 11 $\mu\text{g}/24$ hours. During the first five days of life no difference could be detected between the full term infant and the premature infant. The glucocorticoid excretion remained at approximately the same level up to the 10th day of life and after that time there appeared to be a gradual increase in the excretion with advancing age in both groups of babies.

DISCUSSION

As glucocorticoids were found to be present in the urine of the newborn infant, it must be concluded that the adrenal gland is capable of elaborating these hormones at birth. The possibility that these substances might be transmitted through the placenta from the maternal circulation seems doubtful as, in many instances, the urine collection was not begun until the third day of life, also it was not possible to detect any activity in the placenta itself. The urine obtained from infants born prematurely (4 to 8 weeks) also contained small amounts of glucocorticoids and no difference between the full term and the premature infant was observed. In both groups of babies the excretion of glucocorticoids slowly increased with age. According to Benner (1940) growth of both medullary and true cortical tissue proceeds fairly constantly in fetal and post-natal life in spite of the rapid involution of the fetal cortex. As there is a gradual increase in the excretion of glucocorticoids at a time when rapid degeneration of the fetal cortex is occurring, these findings do not support the suggestion that the fetal cortex is elaborating glucocorticoids.

Swinyard (1943) in a cytological study of the adrenal gland of the newborn states that the adult cortex is entirely zona glomerulosa, the zona fasciculata only appearing during the second post-natal week. In studies on the rat adrenal, Deane, Shaw and Greep (1948) have correlated the zona glomerulosa with changes in electrolyte metabolism while morphological and histochemical alterations in the zona fasciculata are correlated with functional tests for the glucocorticoids.

In the newborn infant therefore, glucocorticoids are being elaborated by the adrenal gland before the appearance of the zona fasciculata.

That the adrenal gland of the newborn infant is capable of responding to stress in a manner similar to that observed in adults by Venning and Browne (1944), is indicated by the findings in the two premature babies suffering from atelectasis. In Baby B, there was a marked increase in output of glucocorticoids at a time when the damage was most severe. As the lungs cleared, the excretion of these substances returned to levels found in healthy babies of the same age. At 54 days of age, this baby was excreting approximately twice the average amount of glucocorticoids found at birth.

CONCLUSIONS

The newborn baby excretes small amounts of glucocorticoids.

There is no apparent difference between the amount of glucocorticoids excreted by the full term or the premature infant.

The excretion of glucocorticoids gradually increases with age of baby.

This gradual increase in the excretion of glucocorticoids coincides with the rapid involution of the fetal cortex. This fact does not seem to support the suggestion that the fetal cortex of the newborn is elaborating glucocorticoids.

The adrenal gland of the newborn is capable of responding to severe trauma with an increased output of glucocorticoids.

No glucocorticoids could be detected in placental extracts.

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DESOXYCORTICOSTERONE ACETATE: STUDIES ON THE REVERSIBILITY OF ITS EFFECT ON BLOOD PRESSURE AND RENAL DAMAGE IN RATS¹

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IN PREVIOUS studies it has been reported that the administration of desoxycorticosterone acetate (DCA) to rats, when accompanied by a liberal intake of sodium chloride, leads to cardiac and renal enlargement (Selye, 1940; Green, Coleman and McCabe, 1948; Knowlton, Stoerk, Seegal and Loeb, 1946), renal damage (Selye 1943) and to the development of hypertension (Grollman, Harrison and Williams 1940; Selye and Hall, 1943). The alterations produced by this steroid are much intensified in the presence of pre-existing renal damage. With the experimental conditions employed in this laboratory, hypertension following DCA injections has been observed only in rats previously rendered nephritic by a cytotoxic serum (a rabbit anti-rat kidney serum). In these hypertensive animals, a more rapid progression in the course of the pre-existing nephritic process has been found.

The present study was undertaken in an attempt to determine whether the hypertension in rats given anti-kidney serum and DCA was a phenomenon dependent upon its acceleration of the renal damage, or whether it was due to another effect of the steroid. Were the former assumption correct, discontinuation of the steroid injections, once hypertension was established, should have no significant effect upon the blood pressure. On the other hand, were the latter concept correct, a reversal of the hypertensive levels should occur upon withdrawal of DCA.

An additional facet of the problem was studied at the same time. It has been shown that a normal or high intake of sodium chloride is essential for the effects of DCA to become manifest (Knowlton, Loeb, Stoerk and Seegal, 1947). In view of this, it seemed of interest to determine whether rigid sodium chloride restriction might influence the course of development of the cytotoxic nephritis itself without DCA.

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METHODS

Forty-nine male rats of the Long-Evans strain, ranging in age from 68–83 days, at the start of the experimental period, were divided into three groups of 26, 15 and 8 each. The details of the allocation of the animals are shown in Table I. Groups I and II received a modification of the McCollum diet previously described (Smadel, 1936); in this instance, it was adjusted to contain 1.7 per cent sodium chloride. For Group III, sodium chloride was omitted from the diet. Previous analyses of this low sodium diet have revealed a sodium content of 0.89 m.Eq. per 100 gm. of diet. All animals received tap water to drink, and fluid intake was measured daily.

For two weeks prior to the beginning of the study, and at weekly intervals during the experimental period, blood pressure determinations were made, using a modification of the plethysmographic method, as previously reported (Loeb, Knowlton, Stoerk, and Seegal, 1949).

A qualitative albumin determination was made on the urine of each animal prior to the beginning of the study. During the experimental period, quantitative urine albumin determinations (Shevky and Stafford, 1935) were made at intervals on each rat.

On the first day of the experimental period, all animals were injected intravenously with a rabbit anti-rat kidney serum, prepared as described by Smadel (1936). A pool of sera from rabbits #653 and #663 was used, each rat receiving 0.4 cc. on two successive days, i.e., a total of 0.8 cc. per rat.

Beginning the second day of serum administration, daily injections of 2.5 mg. of DCA² were initiated in the rats of Group I. The DCA preparation contained 10 mg. per cc. in peanut oil; 0.25 cc. was given at each injection, subcutaneously.

In the seventh experimental week, the nephritic DCA treated rats of Group I were subdivided as follows: one-third were sacrificed (Ia), in one-third, DCA administration was continued for an additional nine weeks (Ib), while in the final third, DCA injections were discontinued (Ic) and the animals observed over a period comparable to those in Ib. As a control for Group Ia, half of the nephritic non-DCA treated animals of Group II were sacrificed in the seventh week (IIa), while observations were extended on the remainder of this group (IIb) for an additional nine-week period. At the end of this time, all rats were sacrificed.

At sacrifice, the animals were first anesthetized with ether, then 3 cc. of blood was withdrawn from each rat under oil and the blood pooled according to group. On this pooled blood, determinations of sodium and potassium (Berry, Chappell and Barnes, 1946), chloride (Wilson and Ball, 1928), total protein (Lowry and Hunter, 1945) and serum urea nitrogen (Gentzkow, 1942), were done. An exception was made in the nephritic, non-DCA injected rats sacrificed at seven weeks (IIa). In this group, individual blood samples were analyzed for serum urea nitrogen, and no other determinations were made. At autopsy, these animals were weighed, and organ weights recorded for heart, kidneys and adrenal glands. Sections of these organs were fixed in Zenker's solution for histological study.

² We wish to thank Dr. Kenneth Thompson of Roche-Organon, Nutley, N. J. for his generosity in providing the DCA.

TABLE I. ALLOCATION OF ANIMALS

Group	No. of rats	Diet	Anti-Kidney serum	DCA	Date of Sacrifice
I. a.	8	1.7% NaCl	0.8 cc.	2.5 mg. daily for 7 weeks	7th week
b.	8	1.7% NaCl	0.8 cc.	2.5 mg. daily for 16 weeks	16th week
c.	10	1.7% NaCl	0.8 cc.	2.5 mg. daily for 7 weeks	16th week
II. a.	7	1.7% NaCl	0.8 cc.	None	7th week
b.	8	1.7% NaCl	0.8 cc.	None	16th week
III.	8	Low NaCl	0.8 cc.	None	16th week

RESULTS

During the experimental period, two rats in Group I and two in Group II died. The blood pressures of these animals were comparable to those of others in their respective groups. They have, however, been excluded since three died of intercurrent pulmonary infections, while the fourth died in uremia with a unilateral hydronephrosis. The remaining 45 animals gained weight and completed the experimental period.

Blood Pressure

The detailed data for all groups are presented in Table II; i.e., the weekly average blood pressure reading of all rats in each group is tabulated and the range of values obtained in individual animals is also included. In addition, a portion of the data is presented in graphic form (Graph I).

From this graph and table, it can be seen that all the nephritic animals receiving DCA (Group I) showed a progressive rise in blood pressure for the first nine weeks, the tension then stabilizing at levels of 200 mm. Hg or over. The rise in blood pressure occurs somewhat more slowly in Group Ib, due to the fact that the more hypertensive animals were purposely selected after seven weeks for sacrifice and for DCA withdrawal, leaving the less severely hypertensive rats for continuance of DCA.

Withdrawal of DCA resulted in a fall in the average blood pressure level although, as can be seen in Table II, Group Ic, individual animals showed persistent severe hypertension (three of the nine animals had tensions greater than 190 mm. Hg at sacrifice). The decline in the average blood pressure did not continue downward to normal levels; however, it approached the levels seen in the nephritic Group II animals, which received *no* DCA. Among these latter rats, a moderate degree of hypertension (around 150 mm. Hg) became evident after the tenth week of the experimental period.

TABLE II. TABLE OF WEEKLY BLOOD PRESSURE READINGS

No. of Animals	Group I						Group II				Group III	
	(a) AKS+NaCl +DCA		(b) AKS+NaCl +DCA		(c) AKS+NaCl +DCA (for 7 weeks)		(a) AKS+NaCl		(b) AKS+NaCl		AKS+Low NaCl	
	8		7		9		7		6		8	
Weeks	Aver.	Range	Aver.	Range	Aver.	Range	Aver.	Range	Aver.	Range	Aver.	Range
1	119	110 131	120	114 126	120	93 144	125	121 131	123	115 129	119	109 137
0	123	106 136	119	106 134	118	111 127	126	106 143	123	115 132	124	109 142
Experimental 1	124	111 133	120	110 127	122	111 135	125	110 143	111	109 114		
2	130	105 148	128	118 140	122	114 140	126	114 137			119	112 128
3	152	130 181	143	125 151	153	135 171	126	110 150	109	97 120		
4	168	130 187	171	153 196	166	149 175	132	118 153			118	110 127
5	183	172 192	171	153 196	178	160 204	132	120 146	117	108 120		
6	188	177 202	180	153 199	189	171 231	139	125 151	126	121 129	131	116 145
7	196	183 219	188	157 214	206*	194 219	140	126 153	121	113 126		
8			180	158 197	182	155 211			119	108 129	119	106 133
9			209	177 243	169	134 203			123	116 130	148	139 162
10			213	194 243	189	169 226			128	117 152	140	128 161
11			217	210 243	177	148 210			146	123 176	142	133 150
12			217	208 233	163	130 183			149	128 198	143	136 169
13			216	185 239	160	138 200			146	127 186	140	127 160
14			213	193 233					150	133 173	152	137 165
15			220	208 235	168	130 227			153	126 183	153	137 180
16					176	150 231						

* DCA discontinued after this week.

Finally, it is of interest that in the two groups of nephritic rats given *no* DCA, the blood pressure level was not influenced by rigid sodium chloride restriction (cf. Groups II and III, Table II).

Fluid Intake

The average daily ingestion of water per rat per week is presented in Graph II. The DCA treated animals showed an increased intake compared to both non-DCA injected groups, in the early weeks of the experiment. Discontinuance of the steroid (Ic) was accompanied by a

decrease in water intake. This finding is in harmony with earlier reports in dogs treated with DCA (Ragan, Ferrebee, Phyfe, Atchley and Loeb, 1940). Toward the end of the four-month experimental period, there was an increased intake in all groups, although this was minimal in the nephritic rats of Group III on sodium chloride restriction. The animals in which DCA was continued throughout the study, Ib, ingested the largest amount of fluid, as might be expected.

Albuminuria

No abnormal urinary albumin, as determined by the heat and acetic acid method was observed prior to the injection of anti-kidney serum. Following these injections, all animals developed significant albuminuria (over 5 gm. per liter) (Table III). Because of the marked variation from animal to animal, little importance can be ascribed to the averages presented.

Chemical Determinations

These are summarized in Table IV. The serum sodium determination on pooled sera from DCA treated rats sacrificed after 16 weeks, Ib, was elevated, with an associated slight reduction in serum potassium. This is a characteristic effect of DCA (Ferrebee, Parker, Carnes, Gerity, Atchley and Loeb, 1941). In DCA treated animals sacrificed after seven weeks, no alteration in sodium or potassium was observed. Serum chlorides and serum proteins were essentially similar in all groups. In both groups of animals sacrificed at seven weeks, i.e., the DCA treated rats of Ia and the non-DCA injected animals of IIa, serum urea nitrogen values were just above normal limits. In the animals receiving DCA daily for 16 weeks (Ib), the serum urea nitrogen value was again just above the normal limits, while in the rats in which DCA had been discontinued (Ic), as well as in both groups of animals without DCA (IIb, III), significantly higher levels were observed. Whether or not the difference in serum urea nitrogen levels might be ascribed to differences in filtration rate was not established in this study.

Autopsy Findings

The autopsy findings are summarized in Table III.

Body Weight

The body weights were comparable in the various groups, although, as might be expected, those animals sacrificed at seven weeks weighed slightly less than those sacrificed nine weeks later.

Kidneys

The anti-kidney serum employed in these experiments produced renal enlargement and histological evidence of nephritis in all rats.

The kidney damage involved all portions of the renal tissue, i.e., glomeruli, tubules and interstitial elements. A comparison of the nephritic non-DCA injected rats killed at seven (IIa) and at sixteen weeks (IIb), reveals that with the passage of time, definite progression of the glomerular lesions occurs, although the tubular and inter-

TABLE III. TABLE OF FINAL BLOOD PRESSURE, BODY AND ORGAN WEIGHTS, HISTOLOGICAL FINDINGS, ALBUMINURIA

	Group I			Group II		Group III
	AKS ¹ + DCA ² + 1.7% NaCl diet			AKS ¹ + 1.7% NaCl diet		AKS ¹ + Low NaCl
Date of Sacrifice	(a) 7 weeks	(b) 16 weeks	(c) 16 weeks (DCA stopped after 7 weeks)	(a) 7 weeks	(b) 16 weeks	16 weeks
Number of Rats	8	7	9	7	6	8
Final Blood Pressure						
Average	196	220	176	140	153	153
Range	(183-219)	(208-235)	(150-231)	(126-153)	(126-183)	(137-180)
Body Weight Gm.						
Average	283	290	303	277	281	290
Range	(220-340)	(275-315)	(240-340)	(245-300)	(236-322)	(230-330)
Kidney Weight Gm.						
Average	3.78	3.81	3.71	3.90	3.97	3.35
Range	(3.10-4.46)	(3.23-4.24)	(2.98-5.87)	(2.56-3.94)	(3.26-4.78)	(2.59-4.06)
Animals with Nephritis	8/8	7/7	9/9	7/7	6/6	8/8
Urine Albumin Gm/L						
6th week	54 (13-92)	38 (11-97)	23 (8-40)	27 ³ (22-43)	70 (28-105)	59 (7-135)
15th week	—	18 (10-30)	26 (12-40)	—	41 (21-61)	44 (12-88)
Heart Weight Gm.						
Average	1.22	1.46	1.23	.83	1.01	.94
Range	(1.01-1.49)	(1.23-1.59)	(.87-1.48)	(.81-1.00)	(.93-1.10)	(.81-1.13)
Adrenal Weight mg.						
Average	42	42	43	44	47	47
Range	(32-49)	(39-51)	(39-55)	(36-60)	(41-50)	(39-56)
No. Rats Showing DCA Effect in Adrenals	7/7 ⁴	6/6 ⁴	3/8 ⁴	1/7	3/5 ⁴	0/7 ⁴

¹ AKS = Rabbit, anti-rat kidney serum.

² DCA = Desoxycorticosterone acetate in oil, 2.5 mg. daily.

³ Average of 3 animals.

⁴ One pair of adrenals lost in preparation.

stitial damage was no more severe in the group sacrificed at the later date. Clinically, evidence of renal damage was more marked in the 16-week animals, i.e., blood pressure elevation and retention of urea nitrogen.

In earlier studies, in which less potent cytotoxic sera were employed, DCA definitely enhanced both renal hypertrophy and renal damage. In the present experiments, no augmentation of renal size was observed during steroid administration. Histologically, there was

no striking increase in renal damage which could be ascribed to DCA. It was again noted that the rats sacrificed at four months (Ib) had more glomerular damage than the similarly treated group sacrificed nine weeks earlier (Ia). In view of the fact that no augmentation of renal damage was observed in the DCA treated groups, it is not surprising that no striking differences were noted between these rats and the group in which DCA had been discontinued after the initial seven weeks of the sixteen week experimental period (Ic).

Finally, a comparison of the two nephritic groups which did not receive any steroid injections, reveals that the kidneys of the animals

TABLE IV. TABLE OF CHEMICAL DETERMINATIONS ON POOLED SERA

Group	Serum Na m Eq./L	Serum K m Eq./L	Serum Cl m Eq./L	Serum protein gm./100 cc	Serum urea nitrogen mg./100 cc.	Date of sacrifice
I. a) DCA + AKS + NaCl	143.4	5.0	94.4	6.0	25	7th week
b) DCA + AKS + NaCl	151.6	3.9	95.0	6.1	24	16th week
c) DCA for 7 wks. + AKS + NaCl	145.0	5.6	100.4	5.8	39	16th week
II. a) AKS + NaCl	—	—	—	—	23	7th week
b) AKS + NaCl	143.2	4.9	97.4	5.7	43	16th week
III. AKS + Low NaCl	146.8	4.8	97.8	6.1	44	16th week

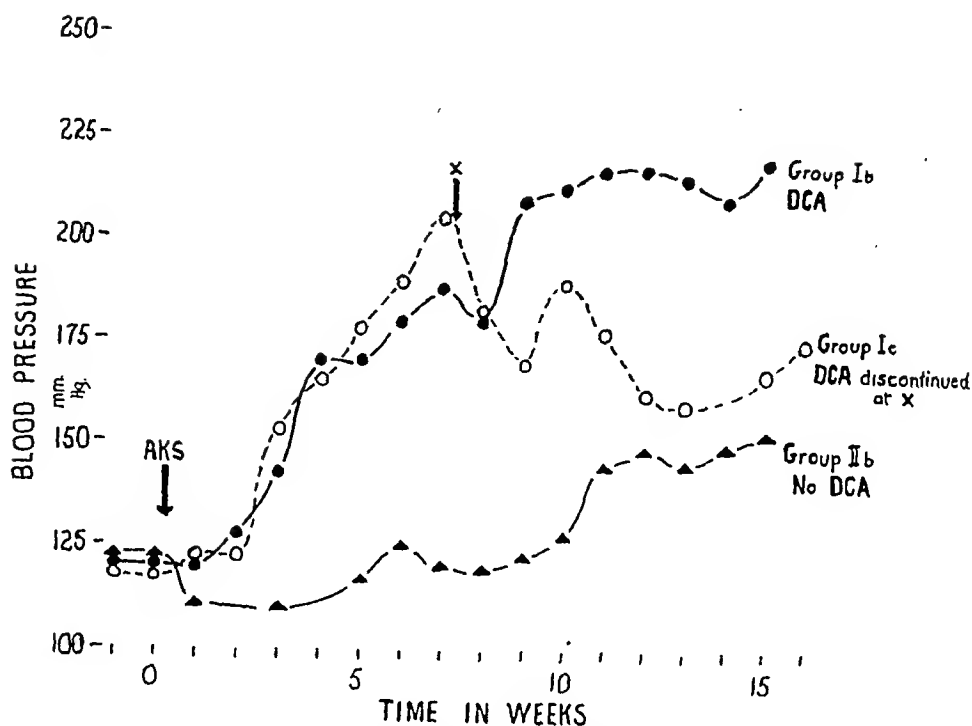
on 1.7% sodium chloride diet (IIb) were slightly heavier than those of the rats on sodium restriction (III). Histologically, however, the evidences of renal damage were not significantly different.

Heart Weights

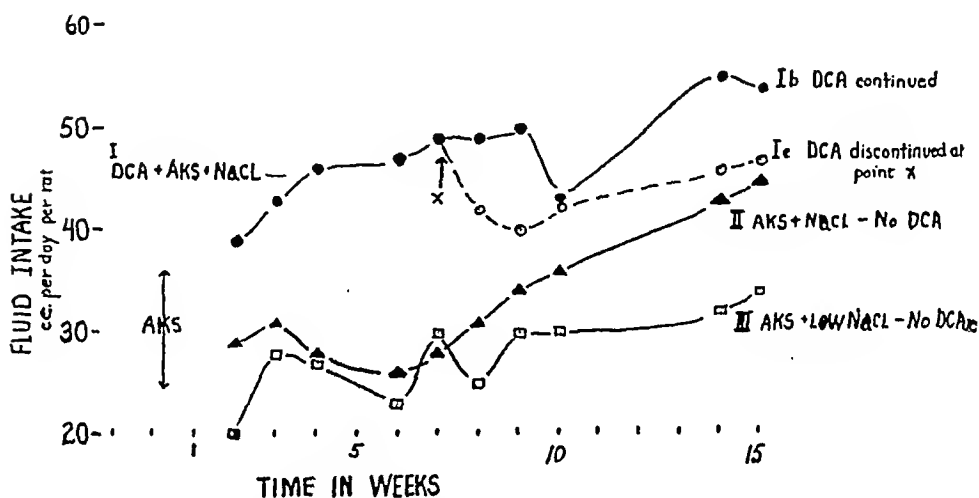
DCA, as previously observed, produced an increase in cardiac size. In those animals (Ic) to which DCA had been given initially but discontinued nine weeks before sacrifice, the heart weights were less than those of animals receiving DCA for the full 16 weeks of the experiment; that is the cardiac hypertrophy did not continue to progress after discontinuation of the steroid. However, it can not be stated that the hypertrophy regressed after withdrawal of DCA, since the heart weights were no smaller than those of rats (Ia) killed at the time DCA was discontinued in Group Ic.

Adrenals

No significant alteration in the size of the adrenal glands occurred in any group. Histologically, the adrenal glands of all nephritic rats receiving DCA at the time of sacrifice showed atrophy of the subcapsular zone as previously reported (Carnes, Ragan, Ferebee and O'Neill, 1941; Greep and Deane, 1947; Knowlton, Loeb, Stoerk and Seegal, 1947). Of the eight adrenal glands examined from the rats in which DCA had been discontinued, only three showed this change, indicating the reversibility of this atrophy. It is noteworthy that in the nephritic group receiving sodium chloride, but no DCA (II), the



GRAPH I.—Average weekly blood pressure readings Groups Ib, Ic, IIb.
 AKS: Rabbit anti-rat kidney serum given to all animals.
 Group Ib: AKS+1.7% NaCl diet+DCA 2.5 mg. daily for 16 weeks.
 Ic: AKS+1.7% NaCl diet+DCA 2.5 mg. daily for first 7 weeks.
 IIb: AKS+1.7% NaCl diet. No DCA.



GRAPH II.—Average daily fluid intake of all groups expressed in cc. per rat per day, charted by week.
 AKS: Rabbit anti-rat kidney serum given to all animals.

adrenal gland of one animal sacrificed at seven weeks and three animals sacrificed at sixteen weeks, showed a similar subcapsular atrophy, the so-called "DCA" change. None of the adrenals of animals from the nephritic group on sodium restriction (III) showed subcapsular atrophy. Indeed, in six of the seven pairs of glands examined, there was distinct hyperplasia of the subcapsular zone.

DISCUSSION

In previous studies in this laboratory (Knowlton, Stoerk, Seegal and Loeb, 1946; Knowlton, Loeb, Stoerk and Seegal, 1947; Loeb, Knowlton, Stoerk and Seegal, 1949), the cytotoxic sera employed have induced minimal evidence of renal damage and no elevation of blood pressure within the periods of observation, which extended up to eight weeks. In the present experiments, the anti-kidney serum proved of considerably greater potency. A severe nephritis was found in the greater majority of rats sacrificed at seven weeks (IIa). Furthermore, in animals observed over a four-month period (IIb), there occurred in addition to histological renal damage, an elevation in serum urea nitrogen terminally and in the majority of the rats, moderate hypertension, while an occasional animal had blood pressures in excess of 175 mm. Hg.

Against this background of extensive renal damage induced by the cytotoxic serum, the accentuating effects of DCA are less evident than those observed previously. However, the increased elevation of blood pressure which ensued when DCA was administered remains impressive. With DCA, the average blood pressure at the time of sacrifice was in excess of 200 mm. Hg, while the nephritic animals without DCA averaged more than 50 mm. Hg lower (Graph I). Bearing in mind that the histological evidence of renal damage was the same in the two groups, one must conclude that the steroid produces an effect on blood pressure not entirely dependent upon demonstrable alterations in renal histology. A further point in favor of this argument is the fact that the hypertensive effect of the steroid is in part reversible upon withdrawal. It is wholly possible that DCA produces its hypertensive effect by means of an alteration in renal blood flow or other functional changes without demonstrable deviations in structure. Why the urea nitrogen in animals with long continued administration of DCA is significantly lower than in all other groups is not apparent at this time. As all animals were on a similar diet and gained similar amounts of weight, the lowered serum urea nitrogen cannot be ascribed to a lowered protein intake in the DCA treated group. Since the serum protein values were similar in all groups, hemodilution can not explain the phenomenon. Furthermore, the increased fluid intake of these DCA treated animals does not seem sufficient to account for the lower urea nitrogen. The low values for serum urea nitrogen in the animals receiving DCA cannot be harmonized

with the lowered blood flow and filtration rate in DCA treated rats reported by Friedman, Polley and Friedman (1948).

The changes in the subcapsular zone of the adrenal gland, seen consistently in DCA treated animals, is apparently not specific for this steroid. In a previous study it was noted that these atrophic changes did not occur following DCA if the sodium intake of the rats was restricted (Knowlton, Loeb, Stoerk and Seegal, 1947). In the present experiments, similar atrophic changes were observed in a number of nephritic animals which had never received DCA, but which were on a relatively high sodium chloride intake (1.7%). It would appear that this is a further instance in which such changes can result from sodium chloride alone, and that DCA acts to intensify the effect of the salt. Furthermore, it is of interest that in the presence of sodium restriction, without DCA, the reverse of atrophy was observed, i.e., actual hyperplasia of this zone of the cortex.

Finally, as to the effects of sodium restriction upon the course of the cytotoxic nephritis, the development of hypertension, urea nitrogen retention and renal damage were not materially altered by rigid reduction of sodium chloride, when a highly potent nephrotoxic serum was employed, as in this study.

CONCLUSIONS

A severe nephritis was produced in rats with the injection of a potent rabbit anti-rat kidney serum.

Hypertension of a moderate degree developed in these nephritic rats independently of variation in sodium chloride intake and without the administration of DCA.

The administration of DCA accelerated the appearance of, and greatly augmented, the hypertension in such nephritic rats.

Withdrawal of DCA, after severe hypertension had been established, resulted in a decrease in blood pressure.

The intensity of the nephritis in this study was not augmented by prolonged administration of DCA, perhaps because of the unusual severity of the process resulting from anti-kidney serum alone.

Subcapsular atrophy of the adrenal cortex, regularly encountered following DCA plus sodium chloride, was observed in some nephritic animals receiving sodium chloride but no DCA. No nephritic animal on rigid sodium restriction showed this change.

Adrenal cortical changes suggestive of hyperplasia of the subcapsular zone were seen in nephritic animals on salt restriction.

ACKNOWLEDGMENTS

The authors wish to express their gratitude to Miss Judith Berg for technical assistance, and to Dr. William Nyhan, Jr., for his help in compiling the data.

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NEW BOOK

THE THYROID AND ITS DISEASES. 2nd Edition 1948. J. H. Means. J. B. Lippincott Co., Publishers. Philadelphia. vii+571 pp. \$12.00.

The first edition of Doctor Means' *Thyroid and Its Diseases* was published in 1937, and the second edition is thoroughly revised to present important advances made in the intervening years.

The plan of presentation remains much the same as in the earlier edition; first anatomical considerations of the thyroid gland are given, followed by a discussion of the nature of its hormone. These chapters cover their subjects in detail, are well documented, and provide instructive reading. The chapter which follows bears on the relations of the thyroid gland to other endocrine glands and is introduced by statements on the similarity of the endocrine system to the nervous system in integrative function. There is a declaration that an attempt will be made to distinguish sharply between well-established facts and uncertain evidence. This attempt is usually successful, but, unfortunately, not regularly so. For example, experimental evidence on the mode of action of thiocyanate and thiouracil on iodine metabolism by the thyroid gland is presented schematically; thiocyanate is shown as imposing a block to the completion of thyroid hormone distal to the uptake of iodine and thiouracil, a block proximal to iodine uptake. Numerous publications have shown that this presentation is incorrect, and indeed, the mode of action of these two substances is such that if "thiocyanate" and "thiouracil" were exchanged in the schema the true situation might be quite accurately portrayed. That this is true is partly recognized in the text although no notice of the contradiction is taken.

The pathology of the thyroid gland in this edition is presented by R. W. Rawson beginning with a review of normal thyroid structure and its alteration by increased need for thyroid hormone and by antithyroid drugs. The section on adenomas follows the classification of Wegelin and that on cancer, of Warren. It is noteworthy that substances such as thiocyanate are said to produce goiters which simulate adenocarcinoma and that Riedel's struma may be mistaken clinically and by microscopic examination for cancer, emphasizing the difficulties of interpretation in this field.

The remainder of the book is concerned with clinical considerations. Symptomatology, methods of examination in thyroid diseases, myxedema, cretinism, and hyperthyroidism are presented in order. Chapters on tumors (by R. W. Rawson), thyroiditis, anomalies, surgery of the thyroid gland (the chapter on Surgery is written by O. Cope), on non-thyroidal diseases treated with thyroid hormone, and on "Facts and Fancy in Matters Thyroid" tend to follow the previous edition, with appropriate revision.

The clinical sections on myxedema and cretinism are delightfully written and stem from a wealth of experience with these disorders. Most clinicians should profit greatly from reading them and will find, in addition to the author's accurate descriptions, well-chosen selections from earlier writings, one from Ord being outstanding to the reviewer. The differential diagnosis

of so-called pituitary myxedema from true myxedema, however, seems not at all in keeping with the otherwise high quality of the section. At least, the reviewer doubts strongly that follicle-stimulating hormone, 17-ketosteroid, and sodium and chloride determinations will often aid in the distinction between them, and in regard to treatment the statement that "an anterior pituitary preparation like chorionic gonadotrophin" may be used in treating pituitary myxedema is distressing on two scores.

As in the first edition a considerable portion of the book is devoted to hyperthyroidism. The classification has been altered; in the first edition the designations of classic exophthalmic goiter or Graves' disease and of toxic nodular goiter or Plummer's disease were used for the clinical separation of toxic goiter, the author, however, expressing doubt as to the validity of the distinctions. The present edition departs from this practice in favor of the term Graves' disease, Plummer's being relegated to another section. Graves' disease, in this edition, is subdivided into the thyrotoxic group, occurring with or without "ophthalmopathy," and into a so-called "hyperophthalmopathic" group, attended by hyperthyroidism, euthyroidism, or hypothyroidism. The incidence of the "hyperophthalmopathic" phase is not made clear but the emphasis on it seems dictated rather more by the author's interests than by the frequency of its occurrence. It is stated without documentation that treatment with irradiation by Roentgen ray or radioactive iodine in cases with severe eye disorders and hyperthyroidism is preferable to thyroidectomy or antithyroid drugs. The objection raised to antithyroid drugs is stated as follows: "They produce what has been called a medical thyroidectomy and are to be avoided for the same reasons as surgical thyroidectomy." Doctor Means avoided a good deal of confusion in this argument by overlooking Dr. Cope's statement in the same book, "The medical therapy of hyperthyroidism with radioactive iodine is essentially a thyroidectomy."

The account of the introduction of antithyroid drugs is sufficiently unusual to warrant quoting: "In our own clinic, interest in the possibility of treating thyrotoxicosis by antithyroid drugs began October 16, 1941. On that day we presented at medical grand rounds a patient who had developed myxedema and hyperplastic goiter while taking potassium thiocyanate as treatment for hypertension. Because of the hypothyroidism that developed as a result of the therapy with this drug, Dr. Earle M. Chapman suggested that potassium thiocyanate might be used in treating hyperthyroidism. Four thyrotoxic patients were, accordingly, treated with this agent. One made a good response, three did not." This would appear an interest heightened in retrospect, for Doctor Means in writing a Medical Progress report for the *New England Journal of Medicine* in October, 1942, made no mention of this interest in antithyroid drugs.

In 1942 Doctor Means stated "the prophecy that surgery will be used less and less in this field (Graves' disease) as the endocrinology becomes better understood," and he noted "Of the treatment of Graves' disease in general it is interesting that in the Thyroid Clinic of the Massachusetts General Hospital we have come full circle in a quarter century. In the years 1919-1922, half the cases were treated by non-surgical methods. In 1932-1935 surgery was employed in 95 per cent. In 1942, half surgical, half non-surgical procedures are being employed." There is in this edition a very sporting

discussion of the relative merits of the several methods now available for treating Graves' disease; and it is interesting to note that in spite of prophecy the circling continues. By 1948 the Thyroid Clinic, having hurdled the radioactive iodine and antithyroid drug obstacles, was running with an easy surgical swing along the 1932-1935 backstretch, a feat clearly establishing its right to lead the field.

Doctor Means has a handsome flair for turning a phrase. He has renamed the old chapter on "Simple Goiter," remarking "Now with the acquisition of new knowledge regarding the action of iodine and the action of goitrogens the matter has become very far from simple. It is almost as if it were the doctors who were simple—not the goiters." The bedclothing difficulties which occur with couples, one of whom has thyroid disease, is referred to as "this thermal variety of connubial maladjustment." "Struma cibaria" and "struma medicamentosa" for goiters due to foods and to drugs seem useful innovations. In regard to "struma cibaria," however, it seems doubtful that even a starving monk could stomach appreciable amounts of rape seed with his cabbage despite the statement to this effect in the text. That "thyropriviac," "to thiouracilize," and "thiouracilization" will prove sufficiently useful to warrant retention is open to question.

Following the main sections of the book there is a chapter on "Thyroid Administration in Diseases of Other Than Thyroid Origin," the use of the hormone for its nonspecific drug action, as it is stated. Doctor Means does not hold to the point of view that the exhibition of small doses of thyroid hormone to euthyroid patients suppresses endogenous hormone production and believes it proved by practice that advantage may be taken of the calorogenic or other action of the hormone and makes an emphatic distinction between the physiologic action in myxedema and the pharmacologic action in conditions not etiologically related to the thyroid. Neither the distinction nor the practical proof is altogether convincing; in regard to the latter the discussion of sterility and habitual abortion provides a case in point. Physicians are enjoined to prescribe thyroid for all cases of sterility not due to local disease regardless of substandard or standard basal metabolism. This recommendation is based on the impressions of many clinicians, but no data are provided which establish the matter; there is no mention of the great difficulties in evaluating results of treatment in sterility and abortion. Undoubtedly physicians are often called upon to do something, that is, to conduct clinical experiments. Unfortunately, reasonable experimental conditions are seldom met; proper selection of cases, recording of data and comparison with control material usually are not made. The final result is that it is often not clear despite years of trial whether patients have benefited from a treatment, or have withstood it.

This volume is clearly outstanding in the thyroid field; the late Dr. Joll's book and several others are now well out-of-date, the late Dr. Hertzler's suffered from its intensely personal nature, and Dr. Crile's new volume is restricted to practical aspects of thyroid disease. It is comprehensive in its scope, and is written with perspective and in a charming, leisurely way. Its judgments are almost always well tempered and balanced, seldom dogmatic. Although this review is critical in tone, it would be ill indeed not to declare the defects minor, conspicuous only by contrast with the general merit of this excellent book.

ANNOUNCEMENT OF THE 1950 MEETING OF THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

The Thirty-Second Annual Meeting of The Association for the Study of Internal Secretions will be held at the Sir Francis Drake Hotel, Friday and Saturday, June 23 and 24, 1950, in San Francisco, California.

The Committee on Local Arrangements is comprised of Dr. Hans Lisser, Chairman and Doctors Leslie L. Bennett, Roberto F. Escamilla, Minnie B. Goldberg, Gilbert S. Gordan, Laurance W. Kinsell.

Hotel accommodations will be difficult to secure on short notice; therefore, members are urged to make their reservations at once. All requests must be addressed to: Dr. William Howard Rustad, American Medical Association (Hotel Committee), Room 200, Civic Auditorium, San Francisco, California.

The scientific sessions will be held in the Empire Room of the Sir Francis Drake, and registration will be on the same floor. The annual dinner will be held in the Empire Room on Friday, June 23 at 7:30 P.M., preceded by cocktails at 6:30 P.M.

Those wishing to present papers, which will be limited to ten minutes, should send title and four copies of an abstract of not more than 200 words, to Edward A. Doisy, M.D., St. Louis University School of Medicine, 1402 South Grand Avenue, St. Louis, Missouri, not later than March 1, 1950. *It is imperative that the abstracts be informative and complete with results and conclusions in order that they may be of reference value and suitable for printing in the program and journals of the Association.* Names of non-members who are co-authors must be followed by the words "by invitation," and the principal degree of each author.

Nominations for the Ayerst, McKenna and Harrison Fellowship, the Schering Fellowship in Endocrinology, and the Squibb and Ciba Awards should be made on special application forms which may be obtained from the Secretary-Treasurer, Henry H. Turner, M.D., 1200 North Walker Street, Oklahoma City 3, Oklahoma, and filed with the Secretary not later than March 15, 1950.

Announcing
POSTGRADUATE ASSEMBLY IN
ENDOCRINOLOGY INCLUDING DIABETES

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Miami Beach, Florida Roney Plaza Hotel April 3-8, 1950

The faculty will consist of prominent researchers and clinicians in the field of endocrinology and metabolic disorders, gathered from the United States and Canada.

The course will be a practical one of interest and value to the specialist and those in general practice. The program will consist of lectures, clinics and demonstrations. Ample time will be given to questions and answers at the end of each session, and registrants are encouraged to contact members of the faculty for individual discussions.

The Roney Plaza, one of Miami Beach's most delightful hotels, offers special convention rates to members of this assembly. This is an unusual opportunity for you and your family to enjoy a pleasant vacation and for you to participate in a highly instructive program of the latest advances in endocrinology and metabolism.

A fee of \$75 will be charged for the entire course and the attendance will be limited to 100. REGISTRATION WILL BE IN THE ORDER OF CHECKS RECEIVED AND WILL CLOSE ON MARCH 3, 1950. Should there be an insufficient number of applicants to fill the course, the registration fee will be refunded immediately in its full amount.

Application for approval of this course has been made to the Veterans Administration. Veterans should make formal application to their local agencies on the appropriate form (1905c or 1950) as furnished by the V.A.

Please forward application on your letterhead together with check payable to The Association for the Study of Internal Secretions, to Henry H. Turner, M.D., Secretary-Treasurer, 1200 North Walker Street, Oklahoma City 3, Oklahoma, before March 3, 1950. Further information and program will be furnished upon request.

Hotel reservations should be made directly with the Roney Plaza Hotel, Miami Beach, Florida, and the hotel advised that you are attending this Postgraduate Assembly.

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ELEVATION OF BIOTIN ACTIVITY IN THE SERUM OF ESTROGEN TREATED CHICKS; RELATIONSHIP TO HORMONE-INDUCED AVIDIN FORMATION IN THE OVIDUCT

ROY HERTZ, F. G. DHYSE, AND WM. W. TULLNER

From the National Institutes of Health, National Cancer Institute

BETHESDA, MD.

CONSIDERABLE information has accumulated concerning the factor in egg-albumen that neutralizes the biological effectiveness of biotin (Hertz, 1946). This factor has been termed "Avidin" (Eakin *et al.*, 1941) and its presence in both the egg and in the genital tract of several species of birds and amphibia has been demonstrated (Hertz *et al.*, 1942). Moreover, the formation of avidin has been found to be associated with ovarian function in the actively laying hen and avidin production has been experimentally induced in the oviduct of the sexually immature chick by the administration of estrogen followed by progesterone (Fraps, Hertz and Sebrell, 1943; Hertz, Fraps, and Sebrell, 1943).

Trager has described the presence of a fat-soluble factor (FSF) in the plasma of the hen which has biotin-like activity for both the chick and for *Lactobacillus casei* (Trager, 1947, a). He noted further that this biotin-like factor is elevated in the plasma of the actively laying hen (Trager, 1948).

Accordingly, we have attempted to ascertain the hormonal factors involved in the mobilization of FSF into the blood stream of the sexually immature chick and to examine the relationship of this effect to the concomitant formation of avidin in the oviduct. Our data indicate: (1) that the administration of estrogen to sexually immature chicks induces a marked increase in the total biotin content of the serum, (2) that this increase is attributable to or associated with a rise in both FSF and biotin remaining after FSF separation (true biotin), (3) that although the combined administration of estrogen and progesterone induces marked avidin formation in the

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oviduct, there is no apparent effect of this avidin-formation on the simultaneously elevated level of biotin activity in the blood.

MATERIALS AND METHODS

Newly hatched New Hampshire Red chicks were fed commercial starting mash and tap water ad libitum. They were maintained in electrically heated battery brooders with a fixed period of 12 hours of artificial illumination each day. Beginning on the 8th day of life, diethylstilbestrol and progesterone were administered separately in 0.2 cc. corn oil subcutaneously each day for 8 days in the doses and combinations indicated in Table I. Control animals were completely untreated and accompanied each experimental group. Twenty-four hours after the last injection the chicks were killed by bleeding from the heart and the plasma, oviducts and other tissues prepared for assay.

The plasma was prepared from the heparinized blood by prompt centrifugation. One volume of plasma was mixed with 10 volumes of 3N H₂SO₄ and autoclaved for 1 hour at 15 lbs. pressure. The lipid fraction was separated from this hydrolysate by the method of Trager (Trager, 1947, b). Both the ether soluble and the aqueous fraction were assayed for biotin activity.

For the avidin determination, the magna of the oviducts were carefully dissected and homogenized in normal saline and the assay carried out by the yeast growth test described by Hertz (Hertz, 1943).

Biotin and biotin-like activity was determined by the microbiological method employing *L. casei* as the test organism (Landy and Dicken, 1942).

RESULTS AND DISCUSSION

The data from a representative experimental series are presented in Table I. It will be seen that the plasma of the untreated chick has

TABLE 1. EFFECT OF STILBESTROL AND STILBESTROL PLUS PROGESTERONE ON BIOTIN LEVELS IN CHICK PLASMA

	Untreated (12 samples)		Stilbestrol (13 samples)†		Stilbestrol plus pro- gesterone§ (8 samples)	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
Biotin Activity* (Fat-Soluble Fraction)	3.8	0.79	15	4.4	11	1.8
True Biotin* (Aqueous Fraction)	1.3	0.22	8.3	1.8	10	1.3
Oviduct Weights (mg.)	29.0	6.6	1091.0	192.0	380	93.0
Avidin†	—		—		+	

* Values expressed as milli-micrograms of Biotin per ml. of undiluted plasma.

† (—) signifies less than and (+) more than 0.1 unit of avidin per gram of oviduct magnum (wet wt. basis). A unit of Avidin is the amount required to inhibit completely the yeast growth supported by one microgram of biotin.

‡ Injected subcutaneously with 0.25 mg. stilbestrol in 0.2 cc. corn oil daily for 8 days.

§ Injected subcutaneously with 0.25 mg. stilbestrol in 0.2 cc. corn oil and 1.0 mg. progesterone in 0.2 cc. corn oil daily for 8 days.

a limited biotin activity and that approximately 75% of this activity resides in the fat-soluble fraction. In the estrogen treated birds, and in the estrogen-progesterone treated birds, the total biotin activity in the plasma is elevated about five-fold. In both instances there is a substantial elevation in biotin activity attributable to the fat-soluble fraction (FSF) and to the non-lipoid residue (true biotin). It will be recalled that estrogen alone has been shown to be incapable of inducing avidin secretion whereas a combination of estrogen plus progesterone uniformly induced avidin formation in the young chick (Hertz, Fraps and Sebrell, 1943). Assays of representative oviducts in this series confirmed these earlier observations (Table 1). Thus, the formation of a potent anti-biotin substance in the albumen-secreting portion of the oviduct is not accompanied by an alteration in plasma biotin-activity in either the lipid or aqueous phase. Moreover, the addition of progesterone to the estrogen had no material effect upon the total rise in plasma biotin-activity.

It should be noted that in accordance with our earlier reports (Hertz, Larsen and Tullner, 1947), the simultaneous administration of progesterone and estrogen substantially inhibits the growth of the genital tract induced by giving estrogen alone (Table 1). It is significant that this inhibition of tissue growth is not accompanied by any depression in plasma biotin-activity, but is accompanied by avidin formation in the oviduct. The role of such metabolites as biotin and avidin in the mechanism of hormone-induced tissue growth remains a challenging enigma. Related observations on the explicit requirement for folic acid in the mediation of hormone-induced tissue growth (Hertz and Tullner, 1949; Kline and Dorfman, 1948) indicate that additional trace factors may play a vital role in this type of tissue deposition.

In addition, the very high biotin content of egg yolk (Kögl and Tönnis, 1936) is not to be considered as simply coincidental with the occurrence of a specific antibiotin substance in the albumen of the egg and in the portion of the genital tract in which the albumen is formed. With the added information that a marked increase in plasma biotin-activity can be effected by estrogenization, one is led to consider that biotin and avidin may have a significant function in the physiology of reproduction.

SUMMARY

Total biotin activity is elevated about five-fold in the blood of the estrogen-treated sexually immature chick. This increase in biotin activity is found in both the fat-soluble fraction (i.e. the FSF of Trager) and in the acid hydrolyzable (or true) biotin. Although simultaneous administration of progesterone with the estrogen induces avidin or antibiotin formation in the oviduct, there is no alteration in the degree of elevation of blood biotin-activity. It is considered

that the hormonal induction of increased levels of biotin activity in the blood and the parallel endocrine stimulation of avidin or anti-biotin formation in the female genital tract suggest that these metabolites may play a significant role in the physiology of reproduction.

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BIOASSAY OF HYPOPHYSEAL GROWTH HORMONE: THE TIBIA TEST¹

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INTRODUCTION

IT HAS been known for some time that pituitary growth extracts stimulate the bony epiphyses of rats and that the increments in the width of the epiphyseal cartilage of hypophysectomized rats as results of growth hormone injections is proportional to the dosage (Li and Evans, 1948). This led Evans *et al.* (1943) to propose a simple bioassay method for estimating growth hormone potency. It was found that the method is highly sensitive and simple as compared to other techniques. In this paper we attempt to standardize the procedure and apply it to the assay of pure and crystalline growth hormone.

PRELIMINARY EXPERIMENTS

Prior to the standardization of the test procedure, certain experiments were done varying the route of administration, the duration of the test period, and the frequency of injections of hormone. In these experiments, both tibiae were removed 24 hours after the last injection, fixed in acetone, and stained immediately with silver nitrate, as described below. The width of the cartilage plate was measured with a calibrated eye piece micrometer and the mean width and the standard error for each group were determined.

Effect of the Mode of Injection. A comparison of the effect of subcutaneous, intraperitoneal and intravenous administration of growth hormone was made in two series of animals. In the first experiment, two groups of female rats, 6 weeks old and 2 weeks post hypophysectomy, received a single daily injection of 0.015 mg. growth hormone for 4 days, subcutaneously and intraperitoneally respectively. There was no significant difference between the mean of the proximal epiphyseal cartilage of the tibia in the two groups (Table 1). In second experiment, 20 male rats, 9-12 weeks old and 3-6 weeks post hypophysectomy were divided into 4 comparable groups. Three groups

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TABLE 1. THE EFFECT OF VARYING THE ROUTE OF ADMINISTRATION OF GROWTH HORMONE ON THE RESPONSE OF THE PROXIMAL EPIPHYSEAL CARTILAGE OF THE TIBIA OF THE HYPOPHYSECTOMIZED RAT

Group	Daily dose	Duration of inj.	Cartilage plate response			
			Subcutaneous	Intraperitoneal	Intravenous	Control†
	micro-grams	days	miera	miera	miera	miera
Female rats, 6 wks. old, 2 wks. po.	15	4	235.0 ± 10.3 (7)*	247.0 ± 12.0 (7)	—	159.5 ± 3.0 (34)
Male rats, 9-12 wks. old, 3-6 wks. po.	20	3	135.0 ± 15.6 (5)	143.5 ± 9.2 (5)	131.8 ± 5.7 (5)	93.8 ± 4.4 (5)

* Mean ± standard error; number of animals in parentheses.

† Controls received saline intraperitoneally.

received a single daily injection of 0.020 mg. of growth hormone, for 3 days, subcutaneously, intraperitoneally and intravenously (by tail vein) respectively. The fourth group received saline. Again there was no significant difference between the mean responses of the treated groups (Table 1).

Effect of the Period of Injection. A total of 0.06 mg. of growth hormone was given intraperitoneally in one day, or in divided daily doses for 2, 3 and 4 days. Female rats, 6 weeks old, 2 weeks post hypophysectomy, were used. The results are presented in Table 2. It was noted that this dose of growth hormone administered over 1 or 2 days did not allow a maximum reaction. From the initial studies it appeared that there was no significant difference between the response of the cartilage plate to 0.06 mg. growth hormone when given for either 3 or 4 days. Standard curves were obtained, therefore, with increasing amounts of hormone from 0.02 to 0.2 mg., administered over periods of 3 or 4 days (Table 2). In the 3 day series it was found

TABLE 2. EFFECT OF VARYING THE DURATION AND FREQUENCY OF ADMINISTRATION OF GROWTH HORMONE ON THE RESPONSE OF THE PROXIMAL EPIPHYSEAL CARTILAGE OF THE HYPOPHYSECTOMIZED RAT

Total dose	Cartilage plate response*				
	1 day	2 days	3 days	4 days	5 days
micro-grams	miera	miera	miera	miera	miera
10				212.5 ± 3.7 (23) [197.0 ± 6.4 (4)]‡	214.5 ± 3.2 (4)
20			211.8 ± 12.3 (5)	219.0 ± 6.8 (14)	
40			282.5 ± 7.1 (4)	265.0 ± 8.0 (11) [244.8 ± 6.1 (4)]	281.5 ± 7.1 (4)
60	175.4 ± 7.2 (5)†	227.2 ± 6.0 (5)	262.0 ± 4.0 (10)	271.0 ± 5.6 (10)	
100			296.0 ± 10.9 (4)	319.2 ± 8.5 (4) [294.5 ± 7.9 (4)]	299.5 ± 7.0 (4)
120			261.1 ± 9.1 (5)	313.9 ± 4.1 (29)	
200			286.0 ± 15.0 (9)	336.2 ± 11.2 (4) [317.8 ± 14.6 (4)]	320.0 ± 11.5 (5)

* Single intraperitoneal injection was given daily except as noted.

† Mean ± standard error; number of animals in parentheses.

‡ Figures in brackets represent the response to the same total dose of growth hormone administered in two daily injections.

that the response "plateaued" at dosage levels of 0.04 to 0.06 mg., and above this dose level did not reach the maximum increase as obtained in the 3 day series. Another group of animals were given increasing doses of growth hormone from 0.01 to 0.2 mg. divided into five equal doses administered intraperitoneally once daily for five days. These results did not differ materially from the 4 day injection series.

Effect of the Frequency of Injection. A group of female rats, 6 weeks old and 2 weeks post hypophysectomy, received increasing amounts of growth hormone from 0.01 to 0.2 mg., intraperitoneally, in divided doses twice daily (at 8 a.m. and 4 p.m.) for 4 days. The mean responses are summarized in Table 2, Col. 5. There was no significant difference in the response between administration of the hormone once daily or twice daily.

From these data it is apparent that a single daily intraperitoneal injection, over a period of 4 days, was a satisfactory plan for the routine bioassay of growth hormone.

THE ASSAY PROCEDURE

Female rats of the Long Evans strain were hypophysectomized at 26-28 days of age and injections of growth hormone were begun after a postoperative period of 12-14 days. The animals were maintained on Stock Diet XIV² prior to operation, supplemented by wet Diet I³ postoperative. A pure growth hormone preparation, prepared as described by Li *et al.* (1944, 1945), was used throughout. For each assay, a small amount of the hormone was carefully weighed and dissolved in 20 cc of saline, to which was added one drop of 1N NaOH. From this stock solution, appropriate dilutions were made in normal saline. The animals received 0.5 cc of the growth hormone solution or of saline (controls), once daily intraperitoneally for 4 days. Twenty-four hours after the last injection, the animals were sacrificed with ether or chloroform, one or both tibias were dissected free from soft tissues and split with a sharp razor in the mid-sagittal plane. The bone halves were either stained immediately or fixed in 10% neutral formalin. The staining procedure is as described by Evans, Simpson, Marx and Kibrick (1943). Briefly, the bone halves are washed in water one half hour, immersed in acetone at least 1 hour, and washed in water again for $\frac{1}{2}$ hour. They are then placed in freshly prepared 2% silver nitrate for 1 to 2 $\frac{1}{2}$ minutes, rinsed once in water, and while under water, exposed to a strong light until the calcified portions appear dark brown. They are then immersed in 10% sodium thiosul-

² Stock Diet XIV consists of 5% casein, 68.5% wheat, 10% fish meal, 10% alfalfa leaf meal, 1.5% NaCl, and 5% fish oil.

³ Diet I is slightly modified from McCollum's formula: 67.5% whole wheat, 15% casein, 10% whole milk powder, 0.75% NaCl, 1.5% CaCO₃, 5.25% hydrogenated vegetable oil, and a concentrate of fish oil in amount to give 19 U.S.P. units of Vitamin A and 2.5 A.O.A.C. chick units of Vitamin D per gram of diet.

fate for 25 to 30 seconds, and washed in running water $\frac{1}{2}$ hour. They are stored in 80% ethanol, in the dark.

The width of the uncalcified epiphyseal cartilage is measured under the low power of the microscope using a micrometer eyepiece, calibrated with stage micrometer so that the results may be expressed in micra. A minimum of 8 to 10 readings are made across the section and the results are averaged. The cartilage plate increases in width slightly from anterior to posterior. There is, therefore, an error inherent in the measurement of the mean width. This is illustrated in

TABLE 3. THE ANTEROPOSTERIOR MEASUREMENTS OF THE WIDTH OF THE EPIPHYSEAL CARTILAGE FROM THE RIGHT AND LEFT TIBIA OF A CONTROL AND A GROWTH HORMONE TREATED HYPOPHYSECTOMIZED RAT

Width of uncalcified cartilage, in micrometer units*

	Control		Growth hormone treated†	
	Left	Right	Left	Right
	8	7	16	14
	9	7	18	17
	8	7	19	18
	8	9	19	19
	8	8	20	19
	8	7	20	23
	9	9	20	24
	10	10	21	23
	11	10	23	23
	11	10	20	20
Mean and std. error	9.0 ± 0.40	8.4 ± 0.43	19.6 ± 0.58	20.0 ± 1.02

* 1 micrometer unit 17.8 micra.

† Animal received 160 micrograms growth hormone intraperitoneally in 4 days.

Table 3, where the actual micrometer measurements from a right and a left tibia of a control and a growth hormone treated animal are presented. It will be noted that there is a standard error of 0.4 to 1.0 micrometer unit (7.1 to 17.8 micra) within the measurement of a single tibia. This stresses the importance of an adequate number of readings across the entire width of the tibia in the determination of the individual mean. The variations in the width of the cartilage plate are clearly shown in the photographs of stained tibiae from control and growth hormone treated animals (Fig. 1). In a large series there was no significant difference between the left and the right tibia of an animal, so that for routine work, only one tibia need be taken.

STANDARDIZATION OF PURE GROWTH HORMONE

In this study, a total of 205 animals were used. The data were obtained over a period of several months and the results combined. The data are presented in Table 4. Analysis by the method of Least Squares yielded the following equation: $Y = 139.8 + 79.4 \log X$,

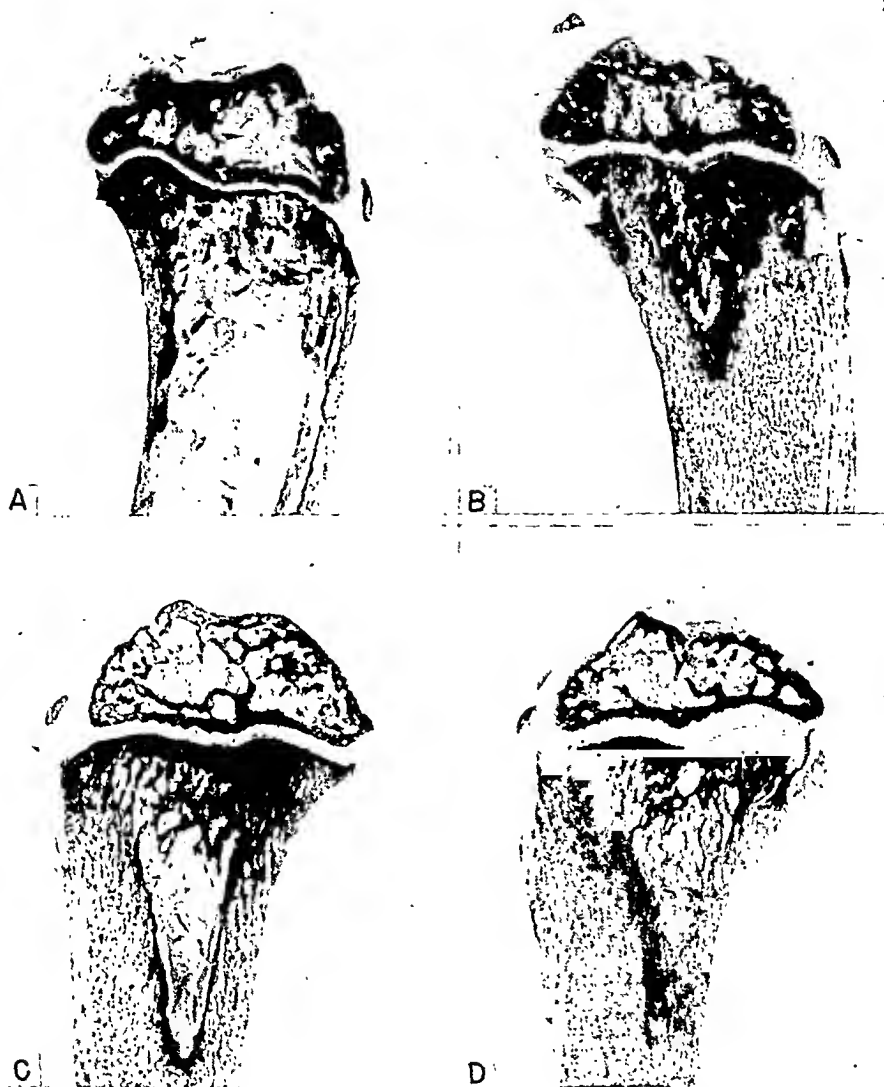


FIG. 1. Effect of Increasing Dose of Growth Hormone on the Proximal Epiphyseal Cartilage of the Tibia of Hypophysectomized Rat in 4 Days. (A) control; (B) 2 micrograms; (C) 16 micrograms; and (D) 120 micrograms.

TABLE 4. THE EFFECT OF PURE GROWTH HORMONE ON THE PROXIMAL EPIPHYSEAL CARTILAGE OF THE HYPOPHYSECTOMIZED RAT

Group number	Total dose	Width uncalcified cartilage	Calculations of "significance"		
			Groups compared	Difference	p†
	micro-grams	miera			
1	0	159.5 ± 3.01 (34)*			
2	2	179.4 ± 3.55 (8)	1 vs. 2	+19.9 ± 6.4	< .01
3	5	188.8 ± 8.62 (12)	2 vs. 3	+11.4 ± 10.45	.27
4	10	212.5 ± 3.73 (23)	3 vs. 4	+23.7 ± 7.9	< .01
5	16	237.6 ± 5.80 (10)	4 vs. 5	+25.1 ± 6.67	< .01
6	20	219.0 ± 6.82 (14)	5 vs. 6	-18.0 ± 9.12	.50
7	40	265.0 ± 8.80 (11)	6 vs. 7	+46.0 ± 10.5	< .01
8	60	271.0 ± 5.57 (10)	7 vs. 8	+ 6.0 ± 10.1	.50
9	80	294.2 ± 6.82 (14)	8 vs. 9	+23.2 ± 7.44	< .01
10	120	313.9 ± 4.09 (29)	9 vs. 10	+19.7 ± 7.7	.02
11	160	338.5 ± 7.17 (8)	10 vs. 11	+24.6 ± 9.1	.01
12	240	321.5 ± 8.40 (10)	11 vs. 12	-17.0 ± 10.7	.10
13	320	341.9 ± 8.83 (8)	12 vs. 13	+20.4 ± 11.6	.10
14	800	360.4 ± 4.86 (10)	13 vs. 14	+18.5 ± 9.1	.05
15	1000	364.5 ± 2.97 (4)	14 vs. 15	+ 4.1 ± 7.3	.60

* Mean ± standard error. Number of animals in parentheses; is corrected for small groups.

† From 't' table, Fisher, 1938.

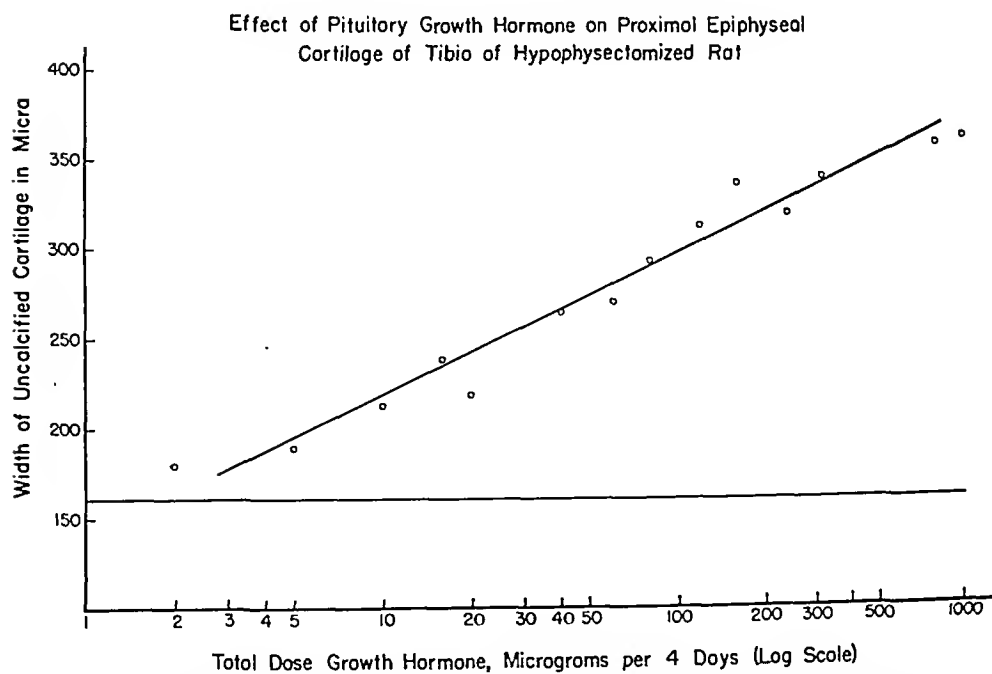


FIG. 2. Effect of Pituitary Growth Hormone on the Width of Proximal Epiphyseal Cartilage of tibia of Hypophysectomized Rat. The Dose-Response Curve.

where Y equals the response of the cartilage plate in micra and X equals the total dose of growth hormone in micrograms. The straight line obtained from this equation, and the means of the responses at each dose level are presented graphically in Figure 2. In order to determine the most satisfactory working range of the test, the data was subjected to the following statistical analysis. At each dose level the mean increment (D) of the cartilage plate response and the standard error of the difference between the given response and the response at the preceding lower dose level (S_D) were calculated for small groups. From the ratio D/S_D a probability value " p " was obtained (Fisher and Yates 1938). These calculations were presented in Table 4, Col. 3, 4, and 5. It will be noted that the responses at the dosage levels of 2 and 5 micrograms were significantly greater than those of the controls, but they were not significantly different from each other. We have arbitrarily considered a total dose of 5 micrograms of pure growth hormone as a minimal effective dose in this series. Between dosage levels of 5 to 120 or 160 micrograms of growth hormone, approximately doubling the dose of hormone produces a significant increment in the response of the cartilage plate. Above a dose level of 160 micrograms, the response levels off somewhat. Thus, we consider the dose range of 5 to 120 micrograms of pure growth hormone satisfactory for the tibia test assay. The actual increase in the width of the proximal epiphyseal cartilage of the tibia of the hypophysectomized rat under the influence of increasing amounts of growth hormone is illustrated in Fig. 1. The changes are apparent even under this low magnification.

Calculations were made of the minimal number of animals necessary to obtain a significant response. The mean increment in cartilage width obtained by doubling the dose of growth hormone between 5 and 160 micrograms is 30 micra. Assuming a desired increment in cartilage width of 30 micra, and considering as significant that the ratio of $D/S_D = 3$, then S_D may be ± 10 micra. If we allow a standard deviation of each response to be ± 20.7 micra (the mean of the series), it can be calculated that the minimum number of animals necessary to obtain a "significant" response is 8.5. In practice, groups of 8 to 10 animals should be used for each assay.

DISCUSSION

The effect of hormones other than growth hormone upon the proximal epiphyseal cartilage of the tibia of the hypophysectomized rat was studied in some detail by Marx *et al.* (1944). It was noted that the daily dose of 5 micrograms of thyroxin administered to a group of 31 animals, produced a mean increment in the width of the cartilage plate of 31 micra. Increasing the daily dose of thyroxin to 15 and 30 micrograms did not further increase the response. Thyrotropic hormone, lactogenic hormone, and testosterone at certain dose levels

may increase the width of the cartilage plate from the control level of 155 micra to a range of 175 to 185 micra, but increasing the dose does not increase the response above these levels. There seem to be a number of substances which cause a slight increase in the epiphyseal cartilage plate of the tibia from 160 to about 190 micra, but increments within this range may not be specific effects. Only pituitary growth hormone will stimulate the cartilage above this level and within limits, the response is directly proportional to the log of the dose of the hormone. On the other hand, the response to a given dose of growth hormone may be augmented by certain hormone combinations. The synergistic action of growth hormone plus thyroxin upon the epiphyseal cartilage has been pointed out by Becks *et al.* (1946).

It was demonstrated by Evans *et al.* (1943) with a partially purified growth hormone preparation, that a smaller total amount of hormone was needed in the tibia test assay than in the body weight test assay. The data presented here with growth hormone confirm this observation. Li *et al.* (1945) have shown that a total dose of 90 micrograms of pure growth hormone was necessary to produce a significant response in the 10 day body weight test. It has been noted above that a total dose of 5 micrograms of pure growth hormone will produce a significant response in the standard tibia test assay. The ratio of the minimum amount of hormone required in the two tests is approximately 1 to 10 or 1 to 20.

Gjeddeback (1948) reviewed the accuracy of some of the methods for the bioassay of growth hormone, using the lambda square test (Bliss and Cattell, 1943) as the index of precision. In this analysis $\lambda^2 = S^2/b^2$, where S is the standard deviation of the variation about the curve, and b the slope of the curve as determined by the method of least squares. From our data, $S = \pm 24.6$, and $b = 79.4$, when $\lambda^2 = 0.0945$. This value compares favorably with the accuracy of the methods reviewed in Gjeddeback's report. In addition, it will be noted from Table 4 that within the dosage range of 5 to 120 micrograms of growth hormone, a dose multiple of 2 produces a significant change in the response. It is of interest to compare the present data, obtained with pure growth hormone, with that reported by Marx *et al.* (1944) who employed a less purified preparation. Analysis of the data of Marx *et al.* by the method of least squares reveals the following equation: $Y = 57.1 + 82.5 \log X$, where Y is the width of the epiphyseal cartilage in micra, and X is the total dose of growth hormone in micrograms. The slope of the line, represented by the constant 82.5 is to be compared with the constant 79.4 obtained from the present data. It is of interest to note that by comparison of the tibia test responses, the pure hormone preparation is approximately 9 times as potent as the one used by Marx *et al.*

SUMMARY AND CONCLUSIONS

A standard procedure for the bioassay of growth hormone, which utilizes the increase in the width at the proximal epiphyseal cartilage of tibia of the hypophysectomized female rat has been outlined.

There was found to be no significant difference between intra-peritoneal, intravenous and subcutaneous administration of the hormone.

Injection periods of 1, 2, and 3 days were found unsatisfactory, whereas 4 or 5 day injection periods produced a satisfactory response. A single daily injection of hormone produced about the same response as injections of the same total dose given twice daily.

The minimum significant increase in the width of the cartilage plate was considered to be 30 micra.

The most satisfactory working range of the curve lay between approximately 190 and 314 micra, which represented the cartilage response to doses of 5 to 120 micrograms of pure hormone. Within this range, and with groups of 8 to 10 animals, approximately doubling a given dose produced a significant increment in the response.

The slope of the curve obtained with pure growth hormone approached that obtained previously with partially purified growth hormone.

The specificity, sensitivity and accuracy of the procedure are discussed.

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THE CHANGES INDUCED IN IODINE METABOLISM OF THE RAT BY INTERNAL RADIATION OF ITS THYROID WITH I^{131} ¹

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DESPITE the present extensive use of radioactive iodine in the treatment of thyroid disease, little is known of the metabolic changes induced within the thyroid gland by the radiation of the trapped I^{131} . Histological changes have, until now, been the principal guide in determining damage to the gland. In the present study, additional criteria are presented, based on the specialized function of the thyroid gland with respect to iodine metabolism. These are: 1) its capacity to remove iodine from plasma, selectively, 2) its capacity to retain iodine, and 3) its ability to release hormonal iodine into the plasma. An attempt has been made to correlate changes in these three functions with the dose received by the gland following the administration of I^{131} . Similar criteria for estimating radiation effects on the thyroid have recently been employed by Skanse (1948).

EXPERIMENTAL

Male rats of the Long-Evans strain, weighing from 200 to 300 gm., were used throughout. They were fed a stock diet composed of 68.5 per cent wheat, 5 per cent casein, 10 per cent fish meal, 1.5 per cent sodium chloride, 5 per cent fish oil, and 10 per cent alfalfa. This diet, which contained 0.3 micrograms of I^{127} per gram, provided each rat a daily intake of 3 to 5 micrograms of iodine. The animals were kept in a temperature-controlled room and had access to food and water at all times.

I. NORMAL RATS TREATED WITH 24, 300, AND 875 MICROCURIES OF I^{131}

Sixty one rats were injected intraperitoneally with a single dose of I^{131} to which no carrier iodine had been added. Each rat received either 24, 300, or 875 microcuries of I^{131} . The rats were sacrificed at intervals of one to eight days thereafter. At the desired interval, the rats were anesthetized by an intraperitoneal injection of sodium pentobarbital

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(20 mg. per rat). Their chests were opened, and 6–8 cc. of blood drawn from the heart directly into a heparinized syringe. Plasma was separated by centrifugation and the protein-bound iodine determined by a method described elsewhere (Taurog and Chaikoff, 1946a, 1948).

The thyroid glands were excised and weighed, and approximately half of a single lobe of each gland was transferred to a fixative for histological study. The remainder of each gland was then weighed and homogenized, with 1 cc. of 10 per cent trichloroacetic acid, in an all-glass apparatus. The homogenate was centrifuged and the precipitate

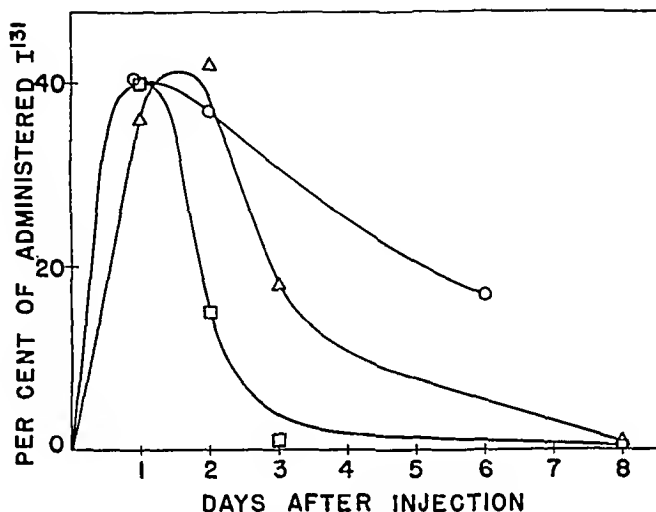


FIG. 1. The uptake of intraperitoneally injected I^{131} by the thyroid glands of rats.

○ 24 microcuries
△ 300 microcuries
□ 875 microcuries

was washed once with 3 cc. of 5 per cent trichloroacetic acid. The combined "supernatants" contained the inorganic iodine fraction of the thyroid. The precipitate (the organic iodine fraction) was dissolved by heating in 1 cc. of 2N NaOH, and diluted to a convenient volume. The chemical iodine content of each fraction was measured as described elsewhere (Taurog and Chaikoff, 1946b). The radioactivity of each fraction was determined by a scale-of-eight Geiger-Müller counter equipped with a thin, mica-window tube.

1. Uptake and Retention of I^{131} by Thyroid Gland in Rats Injected with 24, 300, and 875 Microcuries

The uptake of the injected I^{131} by the glands is shown in fig. 1. The maximum was observed between 24 and 48 hours after injection, and was about 40 per cent of the three dosages.

The rate of loss of the trapped I^{131} from the gland was not the same for the three dosages used. Fig. 1 shows that the greater the radioactivity the of injected dose, the more rapid the depletion of the I^{131} trapped during the first 24-48 hours.

The more rapid loss of I^{131} , observed, when 300 and 875 microcuries, respectively, of I^{131} were injected, indicates that these amounts of radioactivity had injured the gland. Further evidence of damage to the thyroid glands was therefore sought by measuring their contents of total and of organically-bound I^{127} .

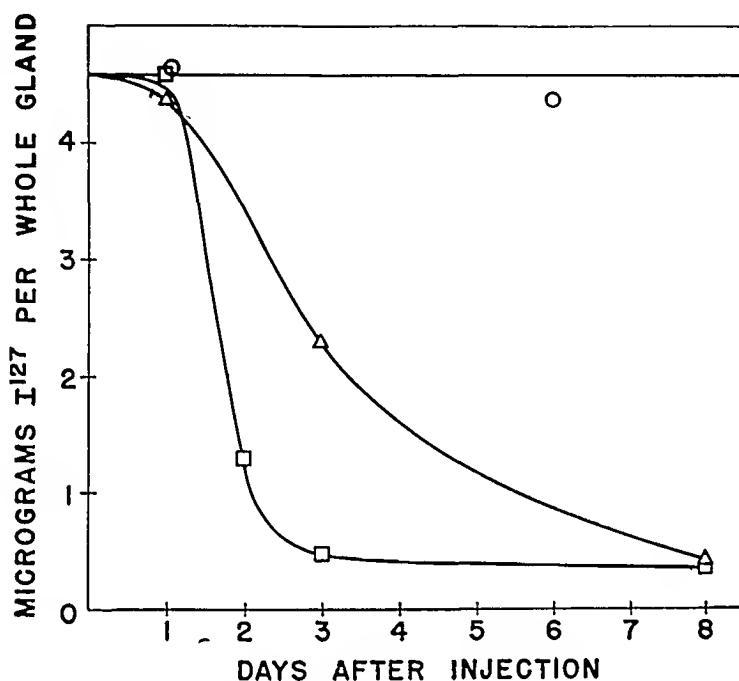


FIG. 2. Loss of chemical iodine (I^{127}) induced in the thyroid gland by injections of I^{131} in rats.

- 24 microcuries
- Δ 300 microcuries
- 875 microcuries

2. Effect of Radiation on the Chemical Iodine (I^{127}) Contained in the Gland

The concentration of organic and inorganic I^{127} in the thyroid glands was measured at five intervals after the injections of the I^{131} —one, two, three, six, and eight days. The results are recorded in fig. 2; each value shown is the average of four observations made on four animals.

No loss of chemical iodine occurred in the glands of rats that had been injected with 24 microcuries of I^{131} for six days. Apparently the dose received by the gland under those conditions does not perceptibly damage the thyroid.

The thyroids of rats injected with either 300 or 875 microcuries did, however, lose considerable amounts of I^{127} . The loss did not occur immediately; none was observed 24 hours after the injection. But by the time two and three days had elapsed, the I^{127} contents of the glands had been reduced to half or less of their initial values, and by the time eight days had elapsed, the I^{127} was practically depleted.

Practically all of this loss in I^{127} involved the organic fraction (Table 1).

TABLE 1. LOSS OF CHEMICAL AND RADIOACTIVE IODINE INDUCED IN THE THYROID GLAND BY INTERNAL RADIATION

Injected dose	Interval after injection	Chemical iodine (I^{127})		Radioactive iodine (I^{131})	
		Organic	Inorganic	Organic	Inorganic
micro-curies	days	micro-grams	micro-grams	per cent of injected dose	per cent of injected dose
24	1	3.7	0.21	39	1.0
24	6	4.1	0.27	16	0.61
300	1	4.1	0.29	35	0.86
300	8	0.55	0.20	0.40	0.23
875	1	4.2	0.32	39	1.0
875	8	0.26	0.10	0.50	0.50

3. The Triphasic Response of Plasma Protein-Bound Iodine to Radiation of the Thyroid Gland

In a recent communication it was shown that, both in its concentration and its rate of formation, protein-bound iodine of plasma responds to experimentally induced changes in thyroid activity in a manner that would be expected of the circulating thyroid hormone (Chaikoff *et al.*, 1947). The injection of thyrotropic hormone increased the concentration of protein-bound iodine of plasma, whereas removal of the thyroids lowered its level. Furthermore, its rate of formation was greatly diminished by thyroidectomy and augmented by injection of the thyrotropic hormone. It is thus evident that protein-bound iodine of plasma is a good index of thyroid activity and for this reason it was used in the present investigation to determine which of the I^{131} doses used had injured the gland.

The changes induced in the concentration of plasma protein-bound iodine by the three doses of I^{131} are shown in fig. 3. No changes occurred in the rats treated with 24 microcuries, an observation that is in accord with the finding of no changes in the I^{127} content of their glands. The values for plasma protein-bound iodine before the injection and on the first, second, and sixth days after the injection of the 24 microcuries were 3.6, 3.6, 3.2, and 3.7 gamma per cent, respectively.

The concentration of plasma protein-bound iodine was reduced as early as 24 hours after the injection of 875 microcuries of I^{131} (fig. 3).

This fall in protein-bound iodine of plasma (referred to here as the initial phase) is the earliest indication so far obtained that the *in situ* irradiation of the thyroid gland had interfered with its metabolic activity.

Plasma protein-bound iodine fell steadily in rats after they had been injected with 300 microcuries. By the time eight days had elapsed, the plasma contained 2.4 micrograms (average) of protein-bound iodine per 100 cc., as compared with an average value of 3.6 gamma per cent before the injections were made.

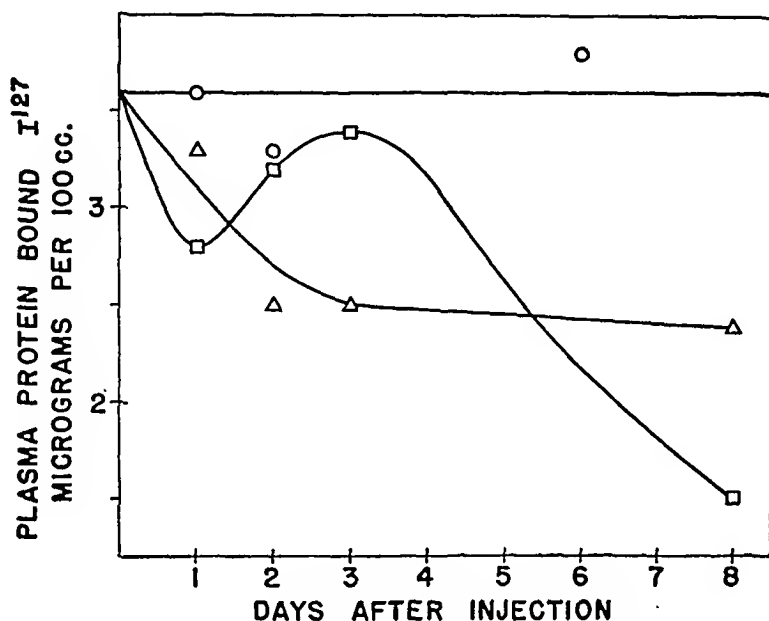


FIG. 3. The response of plasma protein-bound iodine of the rat to injections of I^{131} .

- 24 microcuries
- Δ 300 microcuries
- 875 microcuries

In rats injected with 875 microcuries, an additional change (the second phase) was noted in the level of plasma protein-bound iodine. The initial fall, which undoubtedly was the result of an interference in thyroid activity, was followed by a small, but significant, rise. The time of this rise corresponded with the fall in the concentration of organically bound I^{127} in the gland (shown in fig. 2) and is probably the result of dissolution of thyroid protein induced by the irradiation.

The results of other experiments in which 875 microcuries were injected into rats are shown in fig. 4 and Table 2. It was again observed that an intense radiation of the thyroid gland can induce a pronounced rise in the level of protein-bound iodine, values as high as 7.7 microgram per cent being observed.

The occurrence of this rise in plasma protein-bound iodine at a

time when the gland's iodine is being depleted is also clearly demonstrated in fig. 4.

The third type of response, as shown in fig. 4 and Table 2, consisted of a fall in plasma protein-bound iodine to thyroidectomy levels, i.e., below 1 microgram per cent.

4. Histological Changes

The thyroid tissue was prepared for histological examination as follows. It was fixed in Bouin's fluid, washed for one hour with dis-

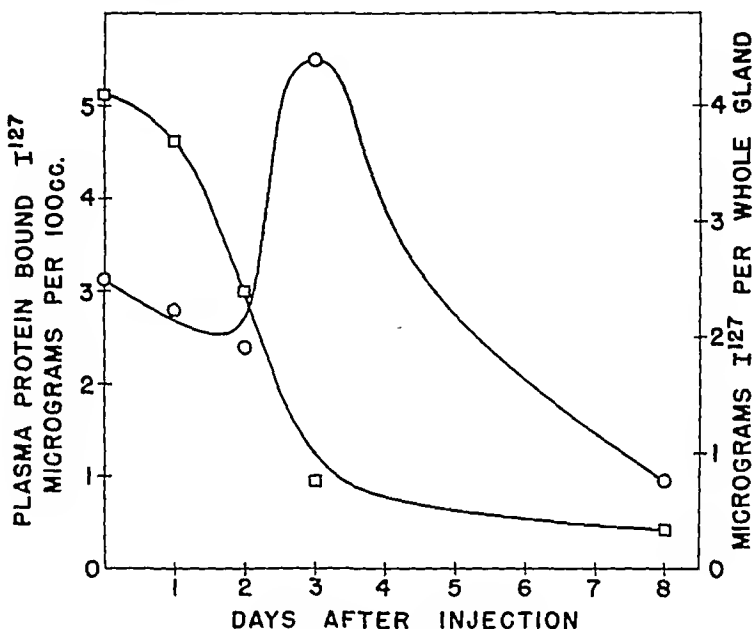


FIG. 4. The relation between the concentration of plasma protein-bound iodine (triphasic response) and the iodine content (I^{127}) of the thyroid gland of rats injected with 875 microcuries of I^{131} .

○ Plasma protein-bound iodine (left abscissa)
 □ Whole thyroid I^{127} (right abscissa)

tilled water, and then dehydrated by three successive washings with dioxane. The tissue was embedded in hard paraffin, cut into sections 5 micra thick, stained with Harris' hematoxylin and eosin, and mounted in clarite X. Kidney and thymus were also examined histologically. The thyroids were examined one, two, three, and eight days after the I^{131} injections.

24 Microcurie Injection—No histological changes were detected in glands of rats injected with 24 microcuries.

300 Microcurie Injection—Extensive epithelial damage was found as early as 24 hours in the thyroid of rats that had been injected with 300 microcuries. In 48 hours, edema, leukocytic infiltration, and fibrin reaction were pronounced, but many apparently undamaged

TABLE 2. THE EFFECT OF THE INJECTION OF 875 MICROCURIES OF I^{131} UPON PLASMA PROTEIN-BOUND IODINE

Rat no.	I^{131} injected	Time after injection	Plasma protein-bound iodine
	micro-curies	days	microgram per cent
	none (controls)	—	3.1*
1	875	3	5.7
2	875	3	3.9
3	875	3	5.1
4	875	3	7.7
5	875	8	<1.0
6	875	8	<1.0
7	875	8	<1.0
8	875	8	<1.0

* This figure represents the average of six measurements on six rats.

follicles were still present. Eight days after injection, the edema was greatly reduced and many active follicles were observed.

875 Microcuries Injection—24 hours after the injection of 875 microcuries of I^{131} , inflammation, edema, and epithelial degeneration were observed. Extensive destruction, with only a few recognizable follicles, was noted by the time 24 hours had elapsed. By the eighth day, only a few isolated, intact thyroid epithelial cells were present, these being in various stages of degeneration. The inflammatory reaction and edema were still evident, and fibrin deposition was prominent.

II. THE IODINE CONCENTRATING CAPACITY OF IRRADIATED THYROID GLANDS

The following experiment was designed to study the uptake of radioactive iodine by thyroid glands previously irradiated with I^{131} . At three and at ten days after a first injection, these rats were injected a second time with I^{131} (see Table 3). The uptake of this second dose was measured at an early interval—six hours. By the use of suitable controls (those rats that received only the first injection), a correction was made for the activity of the first injection that still remained in the gland; this was usually small as compared with that introduced by the second injection.

The results of this experiment are shown in Table 3. In six hours, the glands of untreated rats had accumulated about 40 per cent of the injected I^{131} . The thyroids of rats that had been injected with 30 microcuries suffered no interference in their iodine concentrating capacity.

Three days after the injection of 300 microcuries of I^{131} , the gland lost most of its capacity to concentrate iodine and no recovery had occurred in 10 days.

III. CALCULATION OF THE AMOUNTS OF RADIATION TO WHICH THE THYROID GLAND IS SUBJECTED WHEN RATS ARE INJECTED WITH 24, 300, AND 875 MICROCURIES OF I^{131}

For purposes of the present investigation, only the effects of the beta rays of I^{131} need be considered, because the half-value thickness of tissue for the gamma rays is many times greater than the linear dimensions of the rat thyroid.

In the case of the small rat thyroid, a geometrical factor must be considered in calculating the dose delivered by beta rays of I^{131} because the maximum range of these particles is comparable to the dimensions of this gland. For any case where ionizations occur beyond

TABLE 3. THE UPTAKE OF RADIOACTIVE IODINE BY THYROID GLANDS PREVIOUSLY SUBJECTED TO INTERNAL RADIATION

Rats	Weights of rats	Weights of thyroid glands	First injection			Second injection	
			Amt. of I^{131}	Duration of action	Estimated dose received by the whole gland	Amt. of I^{131}	Uptake by whole gland in 6 hours
	gm.	mg.	micro-curies	days	reps	micro-curies	per cent of injected I^{131}
6	256-270	16.5-21.0	30	10	28,000	73	34.5*
6	250-284	18.5-31.5	none	—	—	73	39.5*
4	240-276	20.0-38.5	300	3	150,000	420	3.5†
3	232-310	19.5-30.0	none	—	—	420	32.4‡
3	210-240	19.0-30.0	300	10	175,000	88	3.5†
3	221-261	27.0-30.0	none	—	—	88	53.2‡

* Average of 6 separate determinations.

† Average of 4 separate determinations.

‡ Average of 3 separate determinations.

the boundaries of a volume of tissue containing radioactive isotopes, a geometrical factor is involved. Marinelli (1948) has dealt with similar factors for gamma ray emitters.

For simplicity, the dose, expressed in roentgen equivalent physical (rep), delivered to the center and to a point near the surface of the rat thyroid is calculated. Geometrical considerations are presented in the addendum.

It is shown that the dose delivered at the center of the gland is

$$D_{\beta^c} = A(1 - e^{-\mu a})$$

and at a point on the surface

$$D_{\beta^s} = A \left[\left(\frac{\mu a - 1}{2\mu a} \right) + \left(\frac{\mu a + 1}{2\mu a} \times e^{-2\mu a} \right) \right]$$

where

μ = absorption coefficient of I^{131} beta particle in cm^{-1} .

a = radius of a thyroid lobe (considered to be spherical) in cm.

The factor A is the accumulated dose in reps originating in the whole thyroid gland. It is given by the following expression (derived below):

$$\frac{N_0(1 - e^{-pt}) \times U \times 2.05 \times 10^5 \times 1.6 \times 10^{-12}}{84 \times w}$$

in which

N_0 = number of radioactive isotopic atoms in the injected dose at zero time.

p = the decay constant, i.e., the fraction of the number of atoms of I^{131} which disintegrate per second.

t = time interval in seconds.

U = average per cent uptake of the injected dose by the thyroid gland during time interval, 0 to t .

w = the weight of the gland in grams.

The terms $(1 - e^{-\mu a})$ and

$$\left[\left(\frac{\mu a - 1}{2\mu a} \right) + \left(\frac{\mu a + 1}{2\mu a} \times e^{-2\mu a} \right) \right]$$

are the geometrical factors for dose at the center and at a point on the surface of the thyroid, respectively.

Let us take the case of a rat injected with 875 microcuries of I^{131} . The total number of disintegrations (given by the expression $N_0(1 - e^{-pt})$) that occurs in such a sample in six days can be obtained as follows:

$$\begin{aligned} N_0(1 - e^{-pt}) &= 875(3.7 \times 10^4) \left(\frac{8 \times 86400}{0.695} \right) [1 - e^{-(0.695 \times 6 \times 86400 / 8 \times 86400)}] \\ &= 13.1 \times 10^{12}. \end{aligned}$$

The number of disintegrations that occurs in the gland itself, however, is dependent upon U , the average uptake of the injected I^{131} . The value for U can be obtained from fig. 1 by measuring the area under the uptake curve and dividing by the time interval. It was found to be 12 per cent of the injected dose.

Thus the total number of atoms disintegrating within the gland in six days = $13.1 \times 10^{12} \times 12/100$ or 15.8×10^{11} .

The emission of each beta particle from I^{131} is accompanied by an average energy output of 0.205 Mev; the energy originating in the whole thyroid gland of the rat will be $15.8 \times 10^{11} \times 0.205 \times 10^6$ electron volts, or 3.2×10^{17} e.v.

The average weight of the rat thyroids used in this experiment

IODINE METABOLISM OF RAT

TABLE 4. ESTIMATED RADIATION DOSE AT THE CENTER AND AT A POINT ON THE SURFACE OF THE THYROID GLANDS OF RATS AT INTERVALS AFTER INJECTION OF 24, 300, AND 875 MICROCURIES OF I^{131}

In- jected I^{131}	1 Day						2 Days						3 Days						6 Days						8 Days					
	Dose delivered			Dose delivered			Dose delivered			Dose delivered			Dose delivered			Dose delivered			Dose delivered			Dose delivered			Dose delivered			Dose delivered		
	At the center		At the surface	At the center		At the surface	At the center		At the surface	At the center		At the surface	At the center		At the surface	At the center		At the surface	At the center		At the surface	At the center		At the surface	At the center		At the surface	At the surface		At the surface
	U^*	reps		U^*	reps		U^*	reps		U^*	reps		U^*	reps		U^*	reps		U^*	reps		U^*	reps		U^*	reps		U^*	reps	
micro- curie	30	150,000	63,000	29	280,000	120,000	22	230,000	120,000	12	290,000	120,000	12	290,000	120,000	12	290,000	120,000	10	290,000	120,000	10	290,000	120,000	10	290,000	120,000	10	290,000	120,000
875	28	46,000	19,000	34	110,000	46,000	32	150,000	63,000	21	170,000	72,000	21	170,000	72,000	21	170,000	72,000	17	180,000	73,000	17	180,000	73,000	17	180,000	73,000	17	180,000	73,000
300	30	4,000	1,800	33	8,900	3,700	33	13,000	5,300	28	19,000	8,000	28	19,000	8,000	28	19,000	8,000	—	—	—	—	—	—	—	—	—	—	—	—

* U is the average per cent uptake of I^{131} accumulated by the rat thyroid gland over the interval studied. See text for further explanation.

was 21.1 mg. Therefore, the average energy released per gram of thyroid tissue was

$$\frac{3.2 \times 10^{17}}{21.1} \times 1000 \text{ e.v., or } 15.0 \times 10^{18} \text{ e.v.}$$

Since $1 \text{ e.v.} = 1.6 \times 10^{-12} \text{ ergs}$, and $1 \text{ rep} = 84 \text{ ergs per gm.}$, then the above number of electron volts are converted to

$$\frac{(15.0 \times 10^{18})(1.6 \times 10^{-12})}{84} \text{ rep, or } 290,000 \text{ reps.}$$

The value for A is shown to be 290,000 reps for the sample calculation.

The geometrical factor for the dose at the center of the gland and at a point near the surface has been shown to be 1.0 and 0.42, respectively (cf. addendum). Therefore, the dose received during six days, at these two points in the thyroid gland of a rat that had been given a single injection of 875 microcuries of I^{131} , was 290,000 reps and 120,000 reps, respectively.

The dose, in roentgen equivalent physical, delivered to these points in the thyroid gland at various time intervals when rats received single injections of 24, 300, and 875 microcuries of I^{131} , was calculated by the above procedure, and the results recorded in Table 4.

DISCUSSION

Table 5 summarizes the various criteria employed in this investigation to assess thyroid function. They are: 1) the histological appearance of the gland, 2) the concentration of plasma protein-bound iodine, 3) the I^{127} content of the gland, 4) the retention of the I^{131} trapped in the gland after the first injection, and 5) the gland's capacity to concentrate a second dose of I^{131} . As judged by all of these criteria, no damage was observed in the thyroid glands of rats ex-

TABLE 5. SUMMARY OF THE CHANGES INDUCED IN THE METABOLIC ACTIVITY OF THE RAT THYROID BY INTERNAL RADIATION WITH I^{131}

Interval after injection when rat was sacrificed	I^{131} injected into rat	Dose at center of the gland	Gland function as judged by the following criteria:				
			Histology	Plasma protein-bound iodine	I^{127} in gland	Retention of first injection of I^{131}	Capacity to concentrate a second dose of I^{131}
days	microcuries	reps					
1	24	4,000	Normal	Normal	Normal	Normal	Abnormal
1	300	46,000	Abnormal	Normal	Normal	Normal	
1	875	150,000	Abnormal	Abnormal	Normal	Normal	
2	24	8,900	Normal	Normal	Normal	Normal	
2	300	110,000	Abnormal	Abnormal	Abnormal	Normal	
2	875	280,000	Abnormal	Abnormal	Abnormal	Abnormal	
3	300	150,000	Abnormal	Abnormal	Abnormal	Abnormal	
3	875	280,000	Abnormal	Abnormal	Abnormal	Abnormal	
6	24	19,000	Normal	Normal	Normal	Normal	
8	30	180,000	Abnormal	Abnormal	Abnormal	Abnormal	
8	875	290,000	Abnormal	Abnormal	Abnormal	Abnormal	Normal
10	30	28,000					
10	300	180,000					Abnormal

aminated six days after an injection of 24 microcuries of I^{131} . Nor was damage observed in glands of rats that were sacrificed 10 days after the injection of 30 microcuries of I^{131} . The dose at the center of the thyroid has been estimated for those two doses (see section III and addendum for sample calculation) and shown to be 19,000 reps and 28,000 reps, respectively (Tables 3 and 4). These amounts of radiation are well within the doses employed in this laboratory, during the past 10 years, for studying iodine metabolism in normal, thyroidectomized, and hypophysectomized rats.

The earliest *chemical* indication of radiation damage to the thyroid was provided by the level of plasma protein-bound iodine, a constituent which, as already pointed out, is synthesized mainly in the gland. A decrease in the concentration of plasma protein-bound iodine was observed 24 hours after the injection of 875 microcuries of I^{131} . This decrease, as judged by histological changes in the gland, is the result of an interference in the formation and/or release of thyroxine by the gland, for it has been shown that plasma protein-bound iodine is largely thyroxine (Taurog and Chaikoff, 1948).

The subsequent rise in plasma protein-bound iodine frequently observed on the second or third day after the injection of 875 microcuries of I^{131} probably results from the release of breakdown products of thyroid tissue into the circulation. Such a rise is not surprising in view of the rapid loss of chemical iodine (i.e., I^{127}) from the irradiated glands as shown in figs. 2 and 4. A similar rise in patients treated with large doses of I^{131} has been observed by Riggs (1948).

It should be noted that a rise in plasma protein-bound iodine was not *always* found in the rats that were injected with 875 microcuries of I^{131} and was not observed at any of the intervals investigated in the rats injected with 300 microcuries. Since thyroid necrosis occurred with both of these doses of I^{131} , it seems reasonable to conclude that a rise in plasma protein-bound iodine occurs only when the damage to the gland is so extensive that the resultant rate of release of thyroid iodine into the circulation exceeds the rates of utilization and excretion. Table 4 shows that a dose at the center of the gland of between 50,000 and 150,000 reps per day is required to produce a rise in protein-bound iodine.

A third type of change in protein-bound iodine was observed eight days after the injection of 875 microcuries of I^{131} . The levels of protein-bound iodine at that time were similar to those observed after thyroidectomy (Chaikoff, *et al.*, 1947).

Retention of the injected I^{131} (fig. 1) is also an indicator of thyroid damage, but a less sensitive one than plasma protein-bound iodine (see Table 5). The percentages of the injected I^{131} found in the glands at the end of 24 hours were approximately the same for the three doses of I^{131} used in this study, but the glands subjected to the larger

doses of radiation (300 and 875 microcuries) failed to retain the accumulated I^{131} beyond the first or second day.

By the time 72 hours had elapsed after the injection of 300 microcuries of I^{131} , it became possible to examine another function of the gland, namely, its iodine concentrating capacity as measured by the six-hour uptake of a *second* injection of I^{131} . It was not feasible to make this measurement before 72 hours had elapsed because the second dose of I^{131} would have had to be several millicuries in order to minimize the radioactivity remaining from the first dose. As judged by the uptake of this *second* injection of I^{131} , it would appear that in three days after a rat receives 300 microcuries of I^{131} , its gland loses the capacity to concentrate iodine.

The effects of internal irradiation with I^{131} on several functions of the thyroid of the five-day-old cockerel have recently been studied by Skanse (1948). However, since this worker used a rapidly growing animal whose tissues would be expected to be more sensitive to ionizing radiations than would those of an adult rat, it is difficult to compare his results with those presented here. Interestingly enough, he reported definite inhibition of the growth of the thyroid of the young cockerel 16 days after the injection of only 10 microcuries of I^{131} , during which time he estimated the glands received 13,000 reps. This is a much smaller dosage than that required to affect the adult rat thyroid, as judged by the criteria presented in Table 5.

Skanse also employed two criteria of thyroid function not used in the present study, namely, the response of the glands to thiouracil and to thyrotropic hormone. He observed a decreased response of the chick thyroid to both these agents after exposure of the glands to approximately 60,000 reps.

SUMMARY

100 adult male rats of the Long-Evans strain were injected intraperitoneally with either 24, 30, 300, or 875 microcuries of carrier-free I^{131} , and were sacrificed at one, two, three, six, eight, or 10 days thereafter.

A method for estimating the dose, in reps delivered to the center and to a point near the surface of the small rat thyroid gland, is presented.

The following criteria were used as indicators of metabolic changes induced in the thyroid glands by the internal radiation of the injected I^{131} : 1) the concentration of protein-bound iodine in plasma, 2) the chemical iodine content of the gland, 3) retention of the initial injection of I^{131} by the gland, and 4) the capacity of the irradiated gland to concentrate a second injection of I^{131} . In addition, all glands were examined histologically.

The earliest *metabolic* indicator of thyroid dysfunction was the concentration of protein-bound iodine in plasma.

Following the injection of 875 microcuries of I^{131} , a triphasic re-

sponse in plasma protein-bound iodine was observed. An initial decrease occurred 24 hours after the injection, at which time 150,000 reps had been delivered to the center of the gland. This initial response was followed by a sharp rise in the level of plasma protein-bound iodine. The highest values were found on the third day after the injection of 875 microcuries. The third, or final, response consisted of a reduction in plasma iodine to thyroidectomy levels.

The injection of 300 microcuries of I^{131} decreased the concentration of protein-bound iodine in plasma. With this dose, however, no subsequent rise in plasma iodine was observed; the level fell steadily.

A change in the iodine (I^{127}) content of the thyroid gland was not observed until two days after the injection of either 300 or 875 microcuries of I^{131} . By this time, the center of the gland had received 110,000 and 280,000 reps, respectively. By the eighth day, the gland's iodine was practically depleted.

The percentages of the initially injected I^{131} present in the gland one day after the injection of 300 or 875 microcuries of I^{131} were the same as those observed in rats injected with 24 microcuries. At the end of two days, however, the thyroid glands of rats which had received 300 and 875 microcuries, respectively, failed to retain the trapped iodine to the same extent as did the glands of rats that had received 24 microcuries. This 48-hour interval corresponds to the time when the chemical iodine was being rapidly lost from the strongly irradiated glands.

Rats were treated with 300 microcuries of I^{131} . Three days later the capacity of their thyroid glands to concentrate a second injection of radioactive iodine was determined. The irradiated glands collected about one-tenth the amount of I^{131} found in the glands of the control (untreated) group.

As judged by the metabolic criteria outlined above, thyroid function is not disturbed in the rat up to 10 days after the injection of 24 or 30 microcuries of I^{131} .

ADDENDUM³

The problem to be considered is the effect of radioactive iodine (I^{131}), distributed throughout the rat thyroid, upon a point at the center of the gland and upon a point near the surface of the gland.

Consider, then, the effect of I^{131} contained in an elementary volume of tissue upon a point, Q , at a finite distant from this volume. The energy absorbed per unit volume of tissue at point Q , due to active material at point P , is equal to

$$\frac{A\mu e^{-\mu r}}{4\pi r^2} dv$$

³ We are indebted to Dr. W. Siri of the Donner Laboratory and to Dr. S. F. Neustadter of the Department of Mathematics for the treatment presented here.

where,

r = distance in cm. from any point P in the elementary volume to the given point Q .

μ = absorption coefficient of I^{131} in cm^{-1} .

A = constant representing the dose delivered to the tissue.

dv = elementary volume.

If, for sake of simplicity, we assume the lobes of the thyroid gland to be spherical in shape and of uniform density as regards the emission and absorption of β -rays, the dose received at the center of the thyroid gland would be

$$D_{\beta^c} = A \int_0^a \int_0^{\pi/2} \int_0^{2\pi} \frac{2\mu e^{-\mu r}}{4\pi r^2} r^2 d\psi \sin \theta d\theta dr$$

or

$$D_{\beta^c} = A(1 - e^{-\mu a})$$

where a = the radius of the thyroid sphere in cm.

For dose at a point on the surface of the sphere

$$D_{\beta^s} = A \int_0^{2a} \int_0^{2\pi} \int_0^{\theta} \frac{\mu e^{-\mu r}}{4\pi r^2} r^2 d\psi \sin \phi d\phi dr, \quad \text{where} \quad \cos \theta = \frac{r}{2a}$$

or

$$D_{\beta^s} = A \left(\frac{\mu a - 1}{2\mu a} + \frac{\mu a + 1}{2\mu a} e^{-2\mu a} \right).$$

The average weight of a single thyroid lobe of rats used in this study was 10.6 mg. The radius of a sphere of such weight can be shown to be 0.135 cm.

An approximation for μ can be made if the density of tissue is assumed to be unity. Thus

$$\mu = \frac{22}{E_{\max}^{1.33}}$$

where E_{\max} is the maximum energy of the beta spectrum. This empirical relation holds only when E_{\max} is larger than 0.5 Mev (Siri, 1948).

In the case of beta particles emitted by I^{131} , $E_{\max} = 0.60 \text{ Mev}^4$ and

$$\mu = \frac{22}{0.60^{1.33}} = 43.5 \text{ cm}^{-1}.$$

⁴ Metzger and Deutsch (1948) have found that there are more than just one E_{\max} of I^{131} as previously supposed. However, for simplicity, only the one value of $E_{\max} = 0.60$ Mev has been used in this calculation.

⁵ This value for the absorption coefficient of the I^{131} beta particle with an $E_{\max} = 0.60$ has been recently verified by an actual measurement of its ionization curve (Siri, personal communication).

The geometrical factors now reduce to

$D_{\beta^c} = 1.0$ (center of the sphere)

$D_{\beta^s} = 0.42$ (point at the surface)

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THE EFFECT OF GROWTH HORMONE ON HEPATIC AND RENAL ACID AND ALKALINE PHOSPHATASES

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CHANGES in the concentration of acid and alkaline phosphatases accompanying growth, in tissues other than bone, have been studied in chick embryos, healing wounds, and tumors (Moog, 1946). It therefore appeared of interest to determine whether or not arresting growth by hypophysectomy or stimulating it by injecting growth hormone alters the concentration of these enzymes in the liver and kidney of rats. Such experiments seemed the more pertinent because the growth hormone characteristically produces storage of nitrogen as protein (Lee and Schaffer, 1934; Young, 1945), and arguments for the implication of phosphatase in protein synthesis have been advanced (Moog, 1946). An increase in hepatic alkaline phosphatase in rats treated with adrenal cortex extracts (Kochakian and Bartlett, 1948) and changes in renal alkaline phosphatase during treatment with steroid hormones (Kochakian and Vail, 1944) also indicate the possibility of such a relationship.

EXPERIMENTAL

The diets, feeding techniques, and animals were those used in preceding papers (Mathies and Gaebler, 1949; Gaebler *et al.*, 1949). The enzyme analyses were carried out according to the procedures outlined by Kochakian (1945), using a substrate pH of 9.1 for the alkaline and 5.4 for the acid phosphatase. Total nitrogen determinations were made on portions of the tissue homogenates by the micro-Kjeldahl procedure, according to Hiller, Plazin, and Van Slyke (1948).

RESULTS

Table 1 summarizes results obtained in the mature male rat. In this experiment only a limited weight gain (17 gm. per rat for the treated group as compared with 4 gm. per rat for the control group) was obtained. This limited response is, however, quite characteristic of the male animal (Evans and Simpson, 1931). Also, no significant change in the concentration of either of the two enzymes determined in liver and kidney occurred. Certainly, it would seem that no pri-

mary relationship could exist between these enzymes and the mechanism of action of the growth hormone. These results are in accord with those of Kochakian and Stettner (1948), who found no change in these enzymes in castrated mice after treatment with growth hormone.

Different results are obtained, however, when the immature, hypophysectomized female rat is used as the test animal. The data

TABLE 1. THE EFFECT OF MASSIVE DOSES OF GROWTH HORMONE ON PHOSPHATASES IN THE LIVER AND KIDNEYS OF MATURE MALE RATS*

Group	Avg. terminal organ wt. in gm.	Avg. phosphatase units†					
		Acid			Alkaline		
		Per gm. tissue	Per mg. total N	Per 100 gm. body wt.	Per gm. tissue	Per mg. total N	Per 100 gm. body wt.
Kidneys							
Treated†	2.79	14.7	0.47	8.2	74.0	2.36	41.5
Control	2.79	13.8	0.43	8.1	64.6	2.02	38.0
Liver							
Treated†	10.96	17.0	0.42	37.4	1.4	0.035	3.1
Control	11.13	15.7	0.38	36.9	1.4	0.034	3.4

* 8-10 month old Sprague-Dawley rats. Rockland rat diet fed *ad lib*. Fasted last 24 hr. period. Five rats per group.

† Given 10 mg. growth preparation (1740 R.U./gm.)/day for 14 days.

‡ Mg. phenol liberated per hr. under the standard conditions.

in Table 2 indicate that rather marked changes in enzyme concentration occur after hypophysectomy in young rats. A comparison of the normal and hypophysectomized controls shows that a considerable decrease in renal alkaline phosphatase has occurred. The basis of calculation did not effect the over-all change, the decrease being roughly 60-70%. The statistical significance of this difference is indicated by the fact that $P < 0.01$ (Fisher, 1948). At the same time, little difference exists between the acid phosphatase assay values. Any changes that occurred could hardly be considered significant ($P < 0.1$; > 0.05). Upon examining the data obtained on the liver, it appears that the acid phosphatase concentration is slightly lower in the hypophysectomized controls than in the normal controls ($P < 0.01$; $t = 6.44$). However, as these animals were not fasted before sacrifice, to avoid the introduction of another variable, the tissue was probably somewhat diluted with glycogen and lipid. Calculation on the basis of activity per mg. of total nitrogen reduces the difference ($P < 0.01$; $t = 5.07$), while calculation on the basis of activity per 100 gm. body weight inverts the difference, hence it cannot be regarded as very significant ($P < 0.4$; > 0.3 ; $t = 1.01$). Hepatic alkaline phosphatase in the hypophysectomized controls has undergone a marked increase in concentration of 2.4-3 fold, depending upon the manner of calculation. The calculated significance for this difference, based on con-

centration per gm. of tissue, is $P < 0.01$. The effects of hypophysectomy, then, can be summarized by saying that the acid phosphatases of the liver and kidneys remain essentially unchanged, while renal alkaline phosphatase is greatly reduced at the same time that hepatic alkaline phosphatase is greatly increased.

Data given in Table 2 show no significant change in the enzymes of either the kidney or liver of normal animals treated with growth hormone. Such findings are in agreement with the results obtained with mature male rats in the previous experiment. Turning to the

TABLE 2. THE EFFECT OF GROWTH HORMONE TREATMENT ON THE RENAL AND HEPATIC PHOSPHATASES OF IMMATURE, HYPOPHYSECTOMIZED, FEMALE RATS*

Group	Avg. terminal organ wt. in gm.	Avg. phosphatase units					
		Acid			Alkaline		
		Per gm. tissue	Per mg. total N	Per 100 gm. body wt.	Per gm. tissue	Per mg. total N	Per 100 gm. body wt.
Kidneys							
Hypophysectomized							
Treated	1.10	19.7	0.65	9.7	128	4.20	63.4
Control	0.88	21.8	0.76	10.8	51.2	1.80	25.3
Normal							
Treated	1.24	19.1	0.64	11.2	128	4.30	74.7
Control	1.34	18.7	0.60	13.0	140	4.50	97.4
Liver							
Hypophysectomized							
Treated	5.39	17.7	0.48	42.8	1.6	0.044	3.9
Control	5.80	14.7	0.43	47.9	2.3	0.067	7.4
Normal							
Treated	4.86	19.1	0.50	43.8	0.88	0.023	2.0
Control	4.62	19.0	0.52	45.5	0.94	0.026	2.3

* Two months old at operation. 5-7 Sprague-Dawley rats per group. Treatment with 1 mg. growth hormone daily per rat (1384 R.U./gm.) for 24 days started 2 weeks after operation. The hypophysectomized controls were fed the diet of Bennett, *et al.* (1948) *ad lib.*, with the remaining groups pair-fed against them. The weight gains of the groups in gms./rat/24 days were: 50, 4, 25, and 5 gms. for the hypophysectomized treated, hypophysectomized control, normal treated, and normal control groups respectively.

effects of growth hormone therapy on the hypophysectomized animals, it is obvious that treatment fully restored the normal complement of renal alkaline phosphatase, calculated on a per mg. of nitrogen basis. Calculation on the basis of activity per 100 gm. body weight reduces the apparent response, due to the low rate of kidney growth as compared with the body weight growth. Hepatic alkaline phosphatase is reduced from its high value in the hypophysectomized animal, although the restoration to normal values is by no means as complete as that obtained with renal alkaline phosphatase.

A similar experiment, of 17 days duration, in which both treated

and control hypophysectomized animals were fed *ad lib.*, while their respective normal control groups were pair-fed against them, gave essentially the same results as in the experiment reported here.

DISCUSSION

When growth is interrupted by hypophysectomy or accelerated by means of growth hormone, alkaline phosphatase concentrations in different organs and tissues do not all change in the same direction. In the tibia, the concentration is low in hypophysectomized animals; it is restored to normal or above by growth hormone, and these changes are readily correlated with arrest and stimulation of skeletal growth (Mathies and Gaebler, 1949). In the kidney the changes are qualitatively the same, but in the liver they are reversed. It follows that each tissue must be analyzed directly, and that changes in alkaline phosphatase concentration of tissue deduced from analysis of serum are not always valid.

It is obviously futile to attempt correlation of changes in hepatic and renal alkaline phosphatase with growth and protein synthesis. Growth is an integral of many reactions. Enzymes catalyze specific reactions, and the functional significance of the reaction which alkaline phosphatase catalyzes differs in different tissues. Presumably, it supplies free phosphate in bone and free glucose in liver and kidney. The decrease in alkaline phosphatase of the kidney and its simultaneous increase in the liver after hypophysectomy might be related to accelerated carbohydrate metabolism in such animals (Bennett, 1936; Russell, 1936; Fisher, Russell, and Cori, 1936). This should increase the demand upon the liver for blood sugar, in the maintenance of which alkaline phosphatase is involved (Cori and Cori, 1938). Whether or not it is compensatory, the rise in liver alkaline phosphatase is in accord with rapid loss of glycogen in fasted hypophysectomized rats (Russell, 1936). Renal alkaline phosphatase is presumably involved in resorption of glucose (Moog, 1946) which should be diminished when oxidation of glucose is accelerated. The decrease of alkaline phosphatase in the kidney could thus reflect diminished need, or it could be part of the general decline in kidney function which White *et al.* (1949) reported in hypophysectomized dogs. However, these investigators found that growth hormone greatly augmented renal clearances in intact dogs, while we find that alkaline phosphatase in the kidney is not increased in intact rats by growth hormone.

The constancy of acid phosphatase concentrations in liver and kidney can be correlated with growth only in the sense that this enzyme behaved like other proteins during arrest or stimulation of growth, and not in any manner indicating a specific relationship. Even in the case of tibia alkaline phosphatase (Mathies and Gaebler, 1949) one can say that observed changes in concentration were

directly related to cessation and stimulation of skeletal growth, rather than to growth hormone, since, in unpublished experiments, no *in vitro* effect of growth hormone on tibia alkaline phosphatase was observed.

SUMMARY

Hypophysectomy of immature, white female rats resulted in an increase in the concentration of hepatic alkaline phosphatase and a decrease in the concentration of renal alkaline phosphatase, while the concentrations of renal and hepatic acid phosphatases remained unchanged. Treatment of these animals with growth hormone partially restored values of hepatic alkaline phosphatase to normal, and completely restored those of renal alkaline phosphatase. The possible relationship of these changes to altered carbohydrate metabolism in the hypophysectomized rat is discussed. The acid phosphatases of both organs remained unchanged in hypophysectomized rats during treatment. Acid and alkaline phosphatases in liver and kidneys were unchanged in the intact rat by growth hormone treatment.

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OXYGEN CONSUMPTION AND PLASMA PROTEIN-BOUND IODINE FOLLOWING ELEMENTAL IODINE INJECTIONS¹

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Dvoskin (1947) reported findings which indicated thyroxine-like action of subcutaneously injected elemental iodine (I_2), such as speeding up the retarded growth rates of thyroidectomized rats, decreasing adrenal gland hypertrophy following thyroidectomy and diminishing the hypertrophy and hyperplasia of the thyroid in thiouracil-treated rats. It seemed probable that these effects were exerted through formation of thyroxine-like substances, possibly at the very site of injection of the I_2 . If so, one would expect to find (1) an elevation in plasma protein-bound iodine (PI) and (2) an increase in metabolic rate as thyroxine or a similar compound was absorbed from the region of production.

These changes actually do result, although the increases in plasma PI are far greater in magnitude than the metabolic effects.

METHODS

The experimental animals used were young adult male and female Sprague-Dawley albino rats, 200 to 350 gms. in weight, the females being consistently lighter than the males. The animals were fed on Purina Laboratory Chow; for the rats on thiouracil, 2 grams of the drug² were thoroughly mixed with a kilo of ground food. Thyroidectomies were performed under ether anesthesia, and the animals were not used until two or three weeks later. A careful search was routinely made at autopsy for gross residual or regenerated thyroid tissue and any likely-looking material was examined histologically. All data reported from thyroidectomized rats are from animals meeting this criterion, but it is acknowledged that the possibility exists that other thyroid residues were undetected.

I_2 requires nearly twice as much iodine in the form of sodium or potassium iodide to put it into a water solution. Preliminary experiments revealed that 6.6 mg. NaI (used to dissolve 3.3 mg. I_2) injected per kilogram of body weight per day for 8 days raised the plasma PI of thiouracil-treated rats from

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² Thiouracil used in these studies was generously furnished by Dr. Stanton M. Hardy of the Lederle Laboratories, Pearl River, N. Y.

1.63 $\mu\text{g.}$ per cent to 4.75 $\mu\text{g.}$ per cent. This complication was removed by dissolving the I_2 in propylene glycol. The only iodine involved was thus the I_2 itself. As the study was progressing, a note appeared from Dvoskin (1947), pointing out the use of liquid petrolatum as a solvent for the I_2 . We continued to use propylene glycol, however, because it was satisfactory as a solvent and could be metabolized by the animals in contrast to mineral oil. Two injection levels were used, 4 mg. and 16 mg. per kilogram per day, each dose in 1 cc. of propylene glycol per kilogram. The desired amount of resublimed I_2 was dissolved with the aid of mild heat and considerable trituration. The solutions were stored in glass-stoppered flasks, great care being taken to avoid any possible contamination of samples of tissues being analyzed for PI or solutions used in the determination.

The injections were made subcutaneously through a fine syringe needle to minimize leaking of the solution. The sites of injection were scattered so that any single location would not be used twice in less than 4 or 5 days. By this means open sores were avoided, although adhesions were often produced.

Oxygen consumption measurements were made with a modified Benedict (1930) multiple-chamber closed-circuit apparatus. The animals were maintained on a special feeding regime (Barker, unpublished) so that they were post-absorptive every afternoon, and repeated determinations could be made without debilitating the animals by repeated fasting.

Protein-bound iodine determinations were run using the procedure described previously (Barker, 1948), modified by the substitution of arsenite for sulfite in the trap of the iodine still (Barker, and Lipner, 1948).

RESULTS AND DISCUSSION

As the most effective presentation of the data, the results of the oxygen consumption studies are shown in graphic form only, as figures 1, 2, and 3. Each curve represents the average of 6 animals treated as indicated with propylene glycol, 4 mg. or 16 mg. I_2 per kilo-

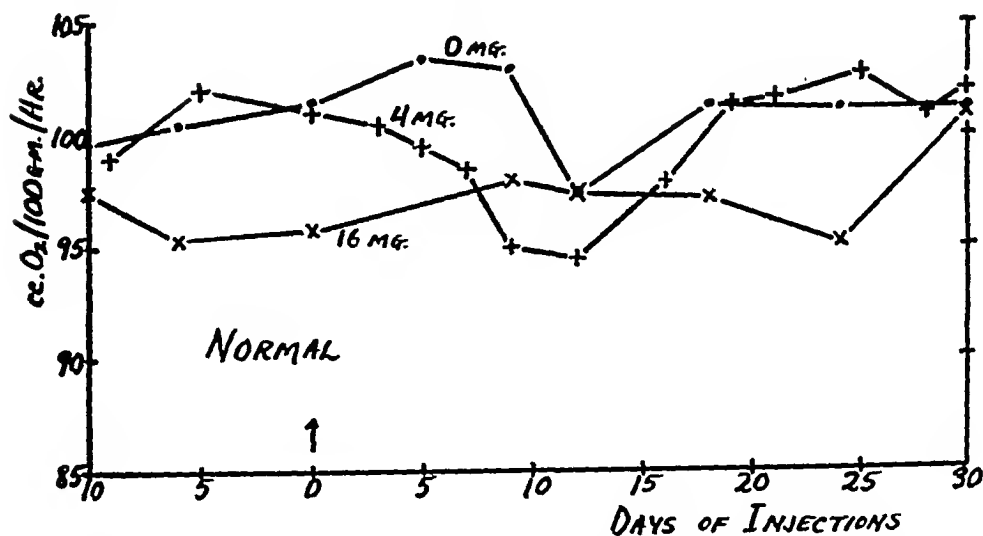


FIG. 1. Oxygen consumption of normal albino rats injected with propylene glycol, 4 or 16 mg. I_2 per kilogram body weight per day in propylene glycol solution.

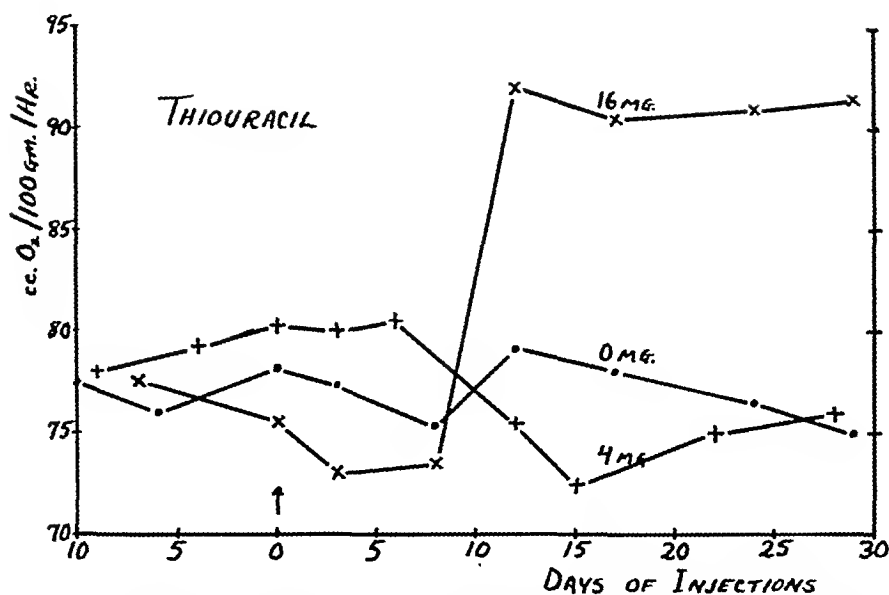


FIG. 2. Oxygen consumption of thiouracil-treated rats injected with propylene glycol, 4 or 16 mg. I₂ per kilogram body weight per day in propylene glycol solution.

gram body weight per day in propylene glycol. Each group is approximately evenly divided between males and females, with no sex differences found in response to the procedures.

Figure 1 reveals no increase in metabolic rate following injection of either 4 or 16 mg. I₂ into normal rats. The only change which seems to follow any sort of pattern is a lowering in the 4 mg. group for the first 10 days, then a return in the next 10 days. The change is small, being of the order of 6 per cent.

The thiouracil-treated animals receiving 4 mg. I₂ per kilogram per day (cf. figure 2) also showed a decrease in oxygen consumption, amounting to about 9 per cent by the fifteenth day. There was only a partial return toward the starting level by the end of the experiment. No ready explanation can be offered for these apparent depressions. In contrast to these fluctuations, a 24 per cent stimulation of metabolism resulted after 12 days on the 16 mg. regime with a latent period of some 8 days. The increase was well maintained for the remaining 17 days of the experimental period.

Examination of the results on the thyroidectomized animals summarized in figure 3 reveals a considerably greater response to the 16 mg. dose, and the first appearance of a metabolic rise from the 4 mg. I₂ per kilogram per day. As can be seen from the graph, the increase resulting from the lower iodine dose was only about 12 per cent, but was consistent. A stimulation of 37 per cent resulted from the 16 mg. injections. Both of these responses were clear by the fifth or sixth

days of treatment and by 10–12 days had reached levels which were sustained to the end of the experiment.

The results of other work, as yet unreported, indicate that the increased oxygen consumption resulting from 4 mg. I_2 per kilogram per day in the thyroidectomized rats is less than that following from the injection of 4 μ g. thyroxine iodine per kilogram per day.³ The elevated

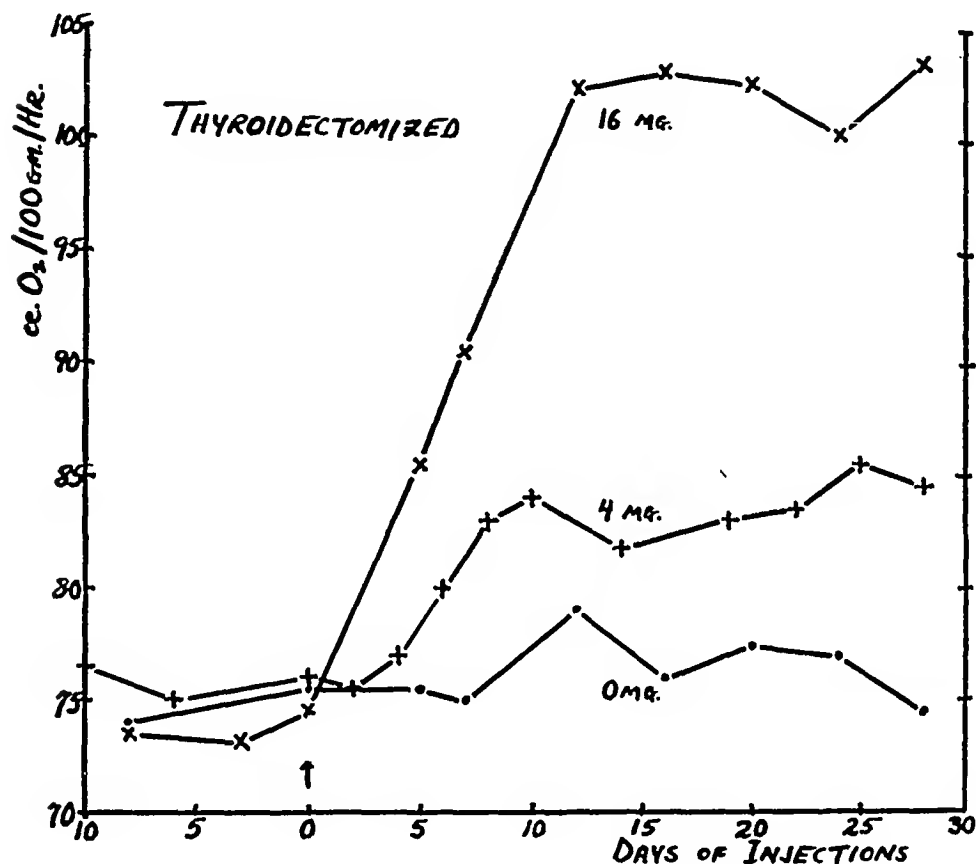


FIG. 3. Oxygen consumption of thyroidectomized rats injected with propylene glycol, 4 or 16 mg. I_2 per kilogram body weight per day in propylene glycol solution.

metabolic level with the 16 mg. dose of I_2 is between those obtained from 8 and from 40 μ g. thyroxine iodine per kilo per day. The only comparable information at present available on thiouracil-treated animals is that 4 μ g. thyroxine iodine per kilogram per day does not change the metabolic rate within two weeks. A repeated dose of 8 μ g. per kilogram per day has no effect on the normal animal. The inference may be drawn from these data that, at least in the thyroidectomized rats, the equivalent of approximately 1 μ g. of thyroxine iodine was formed from each 1 mg. of elemental iodine injected. This may be computed as one-tenth of one per cent efficiency, far less than many of the in vitro iodination processes. However, no special pre-

³ Thyroxine was supplied by Dr. K. W. Thompson of Organon, Inc., Orange, N. J.

cautions to improve the efficiency of thyroxine formation could be taken with the animals, whereas this is possible *in vitro*.

Terminal blood samples were drawn from the animals used for the metabolism studies and the plasma protein-bound iodine (PI) values are shown in table 1. All rats receiving I_2 injections exhibited greatly elevated PI levels of 70 to 120 $\mu\text{g.}$ per cent with 4 mg. I_2 per kilogram per day and 260 to 340 $\mu\text{g.}$ per cent with 16 mg. I_2 . As yet, there is no information concerning the specific nature of the increased plasma PI, whether all thyroxine, which seems unlikely on the basis

TABLE 1. PLASMA PROTEIN-BOUND IODINE IN RATS RECEIVING ELEMENTAL IODINE

	Plasma protein-bound iodine $\mu\text{g.}$ per 100 cc.		
	PG	4 mg. I_2	16 mg. I_2
Controls	5.3	81.9	266
Thiouracil	1.4	72.5	338
Thyroidectomized	1.3	120.0	268

of evidence discussed earlier, or mostly other iodination products, such as monoiodo- and diiodotyrosine. Studies are in progress on this important point.

Although the plasma PI of the thyroidectomized rats receiving 4 mg. I_2 per kilogram per day is higher than the other two groups also on this dose, a similar distinction does not exist at the 16 mg. level, and it is improbable that the greater sensitivity of the thyroidectomized animals can be explained simply on the basis of a greater production of PI. Preliminary data have been reported from this laboratory (Barker and Lipner, 1948) to show that the formation of the PI almost certainly occurs directly at the site of injection of the I_2 probably by a non-enzymatic process similar to the *in vitro* iodination of proteins in general (cf. Reineke, 1946). In such a case, the presence or absence of the thyroid gland would not be expected to exert any influence. The athyroid animal shows a greater metabolic response to a standard dose of thyroxine than does either normal or thiouracil-treated. The responses found in this work can be interpreted most directly along such lines of differing sensitivity of the different animal preparations to thyroxine formed from the injected elemental iodine.

SUMMARY

Groups of normal, thiouracil-treated and thyroidectomized rats have been injected with propylene glycol alone and 4 or 16 mg. elemental iodine per kilogram per day dissolved in propylene glycol, for 30 days.

Study of the metabolic rates during the period of injections showed no effect in the normals of either levels of I_2 . A 24 per cent increase in the thiouracil-treated rats resulted from 16 mg. I_2 , but

no change from the 4 mg. dosage in this group. The lower amount of I_2 produced a 12 per cent, and the greater a 37 per cent elevation in oxygen consumption of the thyroidectomized animals.

Considerable elevations in plasma protein-bound iodine of all animals were produced by both levels of I_2 , the 4 mg. dose causing rises to 70–120 μ g. per cent and 16 mg. to 260–340 μ g. per cent. The increases were approximately the same in all three types of animals, indicating that the thyroid gland was not involved in the production of the protein-bound iodine.

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A STUDY OF THE METABOLISM OF RAT TESTIS IN VITRO¹

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INTRODUCTION

DURING the course of a study of the effect of various gonadotrophins on rat testis it seemed pertinent to correlate previously described changes in chemical morphology (Tepperman and Tepperman, 1947) with the metabolism of the gland *in vitro*. A survey of the literature revealed that Schuler (1944) and Meier and Schuler (1941) had made careful studies of the metabolism of surviving testis. The present study is an outgrowth of experiments that were done for the purpose of confirming an observation made by these authors.

In 1944, Schuler reported that the Q_{O_2} of normal adult testis and that of testicular tissue obtained from weanling rats is about the same in the presence of added glucose. However, when glucose was omitted from the medium, the Q_{O_2} of surviving gland obtained from adult rats was very low, whereas that of the weanling rat's gonad was slightly higher without added substrate than it was in the presence of 0.2% glucose. Schuler attributed this difference in metabolism to the fact that the weanling rat's testis is comparatively "undifferentiated." In this report the hypothesis is developed that a low oxygen uptake in the absence of added substrate is a characteristic of the germinal component of the testis. Moreover, it is suggested that, as the cells of the seminiferous tubules grow to constitute a larger and larger percentage of the gland mass, their distinctive metabolism obscures the metabolic pattern of the non-germinal elements in the gland.

MATERIALS AND METHODS

Albino Farms rats were used throughout this study.

Transplantation of the testis was done with clean, but not aseptic, technique through a mid-line abdominal incision in rats anesthetized with pentobarbital sodium. Stilbestrol pellet implantation was performed according to

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the technique described by Nalbandov and Baum (1948). The 15 mg. stilbestrol pellets were kindly supplied by Mr. Fred Houghton of the Ciba Company. Q_{O_2} was measured over a period of one hour on decapsulated, teased testis tissue in phosphate buffer at a pH of 7.4. Oxygen was the gas phase, the bath temperature was 37.7°C and the shaking speed was 120 per minute. The representative dry weight method was used in the calculation of Q_{O_2} and dry weight included total solids. Glucose was added to the medium where indicated in a concentration of 0.2%. Measurements of respiratory quotient (R.Q.) were made according to the method described by Umbreit *et al.* (1945). The lactic acid method used was that of Barker and Summerson (1941). The chorionic gonadotrophin employed was Follutein, Squibb, and it was supplied through the courtesy of Dr. R. Bates. For the testosterone propionate in sesame oil we are indebted to Mr. Houghton of the Ciba Company.

Means and their standard errors are presented throughout. A difference between means which exceeds three times the standard error of the difference is regarded as significant.

EXPERIMENTAL DESIGN AND RESULTS

1. *Effect of age on metabolism of testis in vitro.*

Results of this experiment are shown graphically in Figure 1. It will be noted that the figures for Q_{O_2} given for 26 gram rats and 300 gram rats constitute good confirmation of the report of Schuler (1944). The intermediate values for 50, 104, 150 gram rats clearly show that, while there is no significant difference in the oxygen uptake

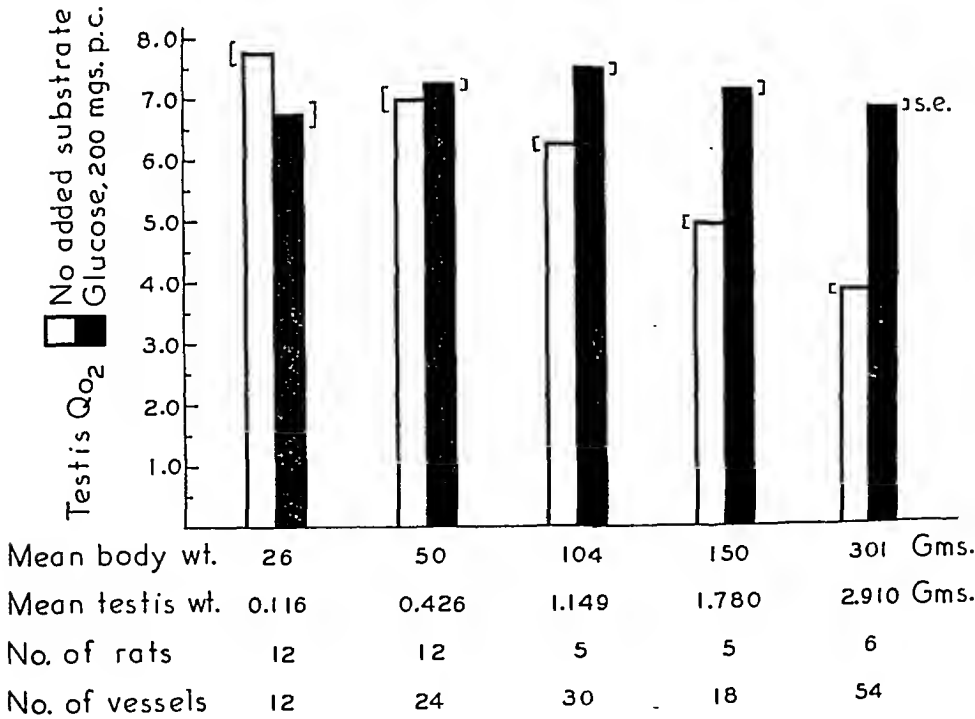


FIG. 1. Effect of age on Metabolism of Testis *in vitro*.

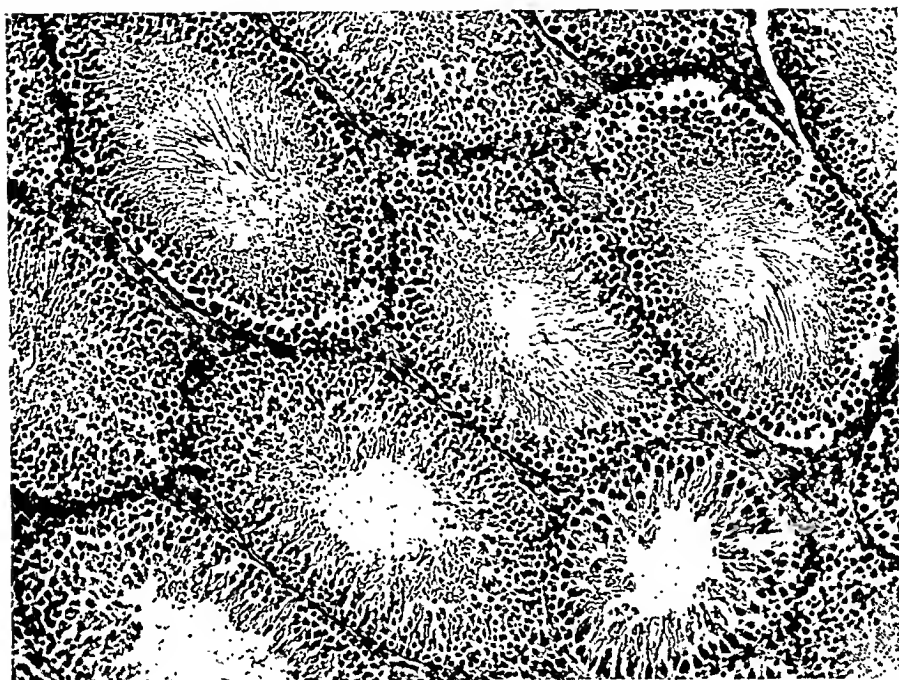


FIG. 2A. Scrotal testis, Experiment 2. X 115.

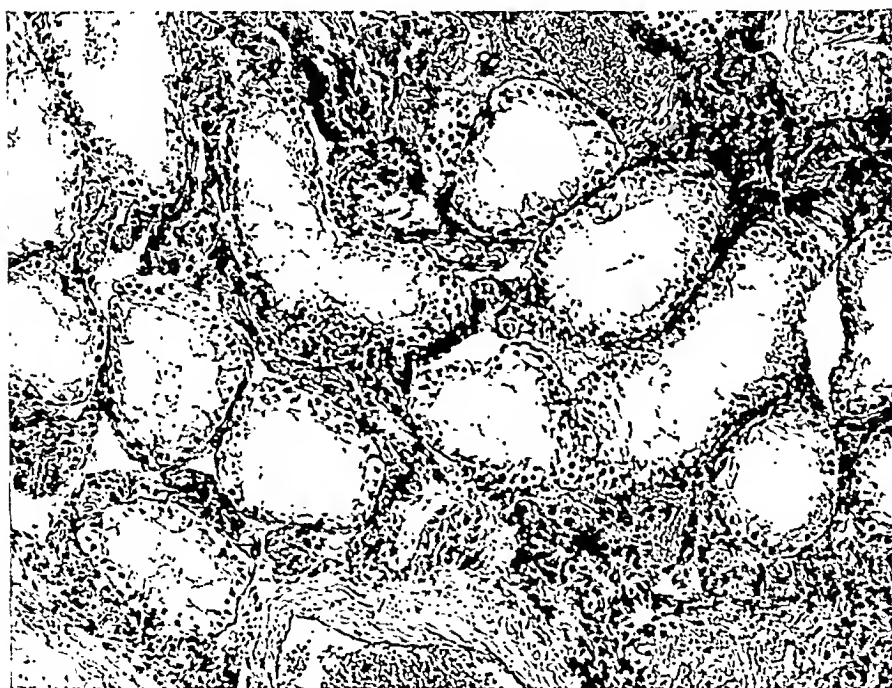


FIG. 2B. Transplanted testis, Experiment 2. X 115.

tubules. Sudan stains of similar preparations showed a very marked increase in Sudanophilic lipid in the cryptorchid glands.

The metabolic pattern of the scrotal and transplanted glands is shown in Figure 3. It is noteworthy that there is no difference between the pattern observed in the scrotal gland and that seen in unoperated animals of similar size. However, the transplanted testis demonstrates a highly significant increase in oxygen consumption in the absence of

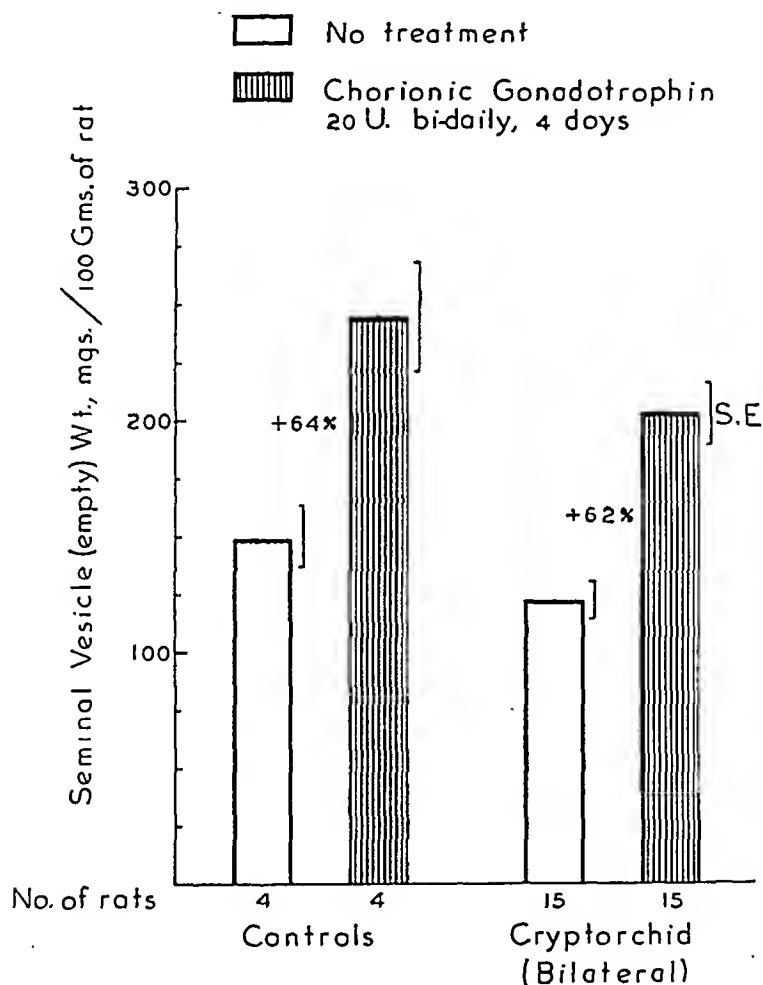


FIG. 4. The response of cryptorchid testes to Chorionic gonadotrophin administration.

added substrate as compared with the scrotal gland. Moreover, this tissue shows a smaller oxygen consumption in the presence of glucose than it does in its absence.

To determine whether or not the transplanted testis is functionally differentiated, a number of bilaterally cryptorchid animals were given chorionic gonadotrophin for four days and the resulting enhancement of androgen output indirectly determined by the seminal vesicle weight technique. The results of this experiment are given in Figure 4. The enhancement of androgen production in cryptorchid animals

given chorionic gonadotrophin was not significantly different from that seen in a representative group of controls. (See Nelson, 1934)

3. *Respiratory quotient and aerobic glycolysis of normal adult, infantile, and cryptorchid testes.*

Since the cryptorchid gland appeared to be less dependent upon the presence of added glucose in the medium than did the scrotal gland, the possibility arose that these tissues might well be meeting their energy requirements by oxidizing different substrates. Therefore,

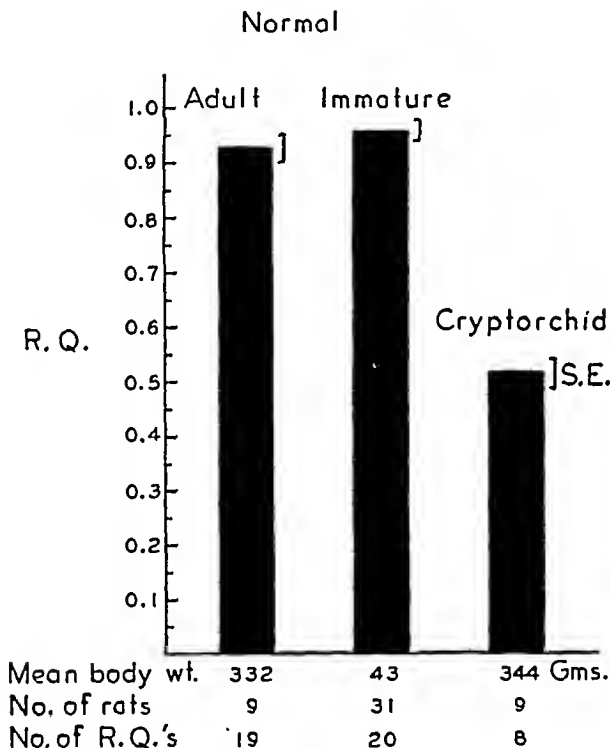


FIG. 5. The respiratory quotient (R. Q.) of normal adult, immature and cryptorchid testes.

R.Q. measurements were made on adult, immature and cryptorchid glands. The results of this experiment are given in Figure 5. There is a highly significant difference between the glucose R.Q. of the cryptorchid gland and that of normal adult gland, but there is no significant difference between the R.Q.'s of immature testis and the adult one.

It is of some methodologic interest that it was technically feasible to make R.Q. determinations on adult and immature testis tissue with little difficulty. However, in the beginning, the R.Q. values for the cryptorchid tissue were extremely variable and many of them were

palpably absurd. For example, in some individual determinations, the initial CO₂ vessel appeared to contain more CO₂ than was produced in the 60 minute CO₂ vessel. This difficulty persisted until a correlation was made in the experimenters' minds between the degree of absurdity of the results and the number of visibly calcified tubules that were seen in the tissue samples. Accordingly, samples were taken from which visibly calcified tubules were rigorously excluded and the respiratory metabolism of these tissues was compared with that of

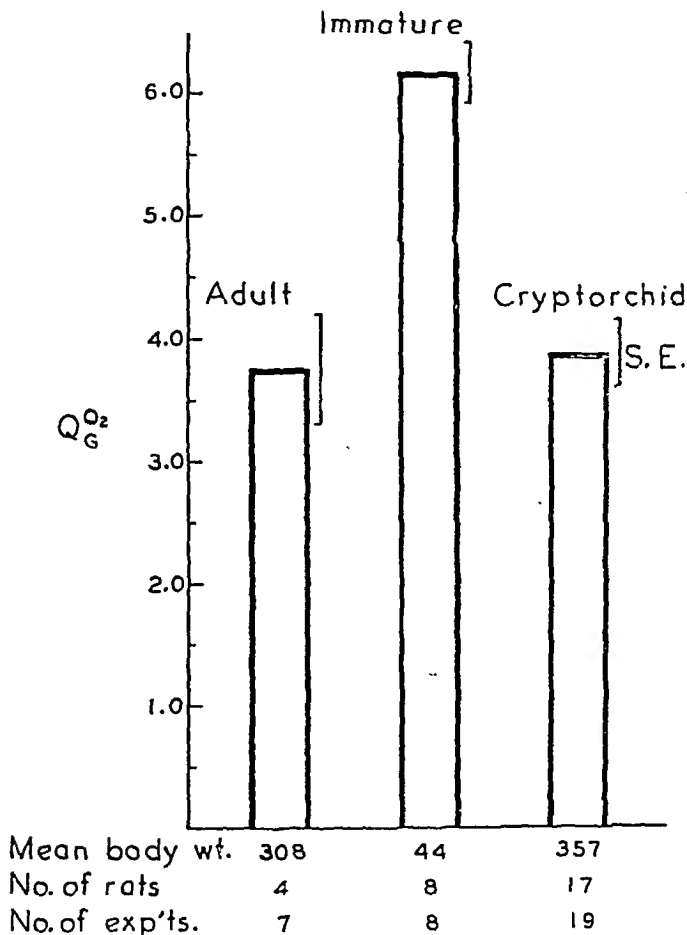


FIG. 6. The aerobic glycolysis of normal adult, immature and cryptorchid testes.

samples which were deliberately contaminated with calcified tubules. The former showed readily reproducible R.Q.'s whereas the latter gave impossibly varied ones. The values reported here for the cryptorchid testis, then, are ones which were obtained from tissue in which there was no visible calcification. It is suggested that the original error was probably based upon the release of CO₂ from CaCO₃ contained in calcified material when the acid was tipped in.

Since Reiss and others (1933) had shown that the aerobic glycoly-

sis of immature rat testis was much higher than that of the adult tissue, an experiment was performed in which this parameter was measured in adult, immature and cryptorchid glands. The results of experiments are shown in Figure 6. In confirmation of previous work (Reiss, 1933), it will be seen that the immature glands have a comparatively high aerobic glycolysis whereas there is no significant difference between the lactic acid production of the cryptorchid gland and that of the adult gland.



FIG. 7A. Normal testis, 150 gm. rat. X 550.

4. *Effects of androgens and estrogens on testis metabolism.*

A group of animals weighing approximately 150 grams each were treated for 7 days with 1 mg. of testosterone propionate per day. At the end of that time they were sacrificed and respiratory metabolism of their testis tissue was compared with that of suitable controls. Histologically there was a striking decrease in the interstitial cell mass which is well shown in Figures 7A and 7B. Whereas there was no significant change in the glucose Q_{O_2} , the testosterone treated animals showed a small but statistically significant decrease in the Q_{O_2} without added substrate (Table 1). The effect of testosterone was greatly magnified when a single experiment was performed on bilaterally cryptorchid animals. In this experiment, as Table 1 clearly shows,

arations, cell counts revealed an approximate ratio of Sertoli cells to interstitial cells of 1:1. If, as Huggins and Moulder (1945) have suggested, Sertoli cells are a source of estrogen, their metabolism might be expected to resemble that of androgen-producing cells rather than that of germinal epithelial cells.

Although there is a certain similarity between the metabolism of cryptorchid and immature testis, Figs. 5 and 6 make it clear that these tissues can be differentiated on the basis of criteria other than the Q_{O_2} with and without added glucose. Immature testis resembles the adult gland in its glucose R.Q., whereas it differs from both adult and cryptorchid tissue in aerobic glycolysis.

The experiment described in Table 1 indicates that a procedure designed to produce atrophy of the androgen-producing cells of the testis may have only a slight effect on the metabolism of whole testis, whereas the same maneuver in bilaterally cryptorchid rats results in a more marked inhibition of oxygen consumption. Conversely, preliminary experiments have indicated that increases in oxygen consumption produced by gonadotrophin injection can be greatly magnified if measurements are made on cryptorchid glands.

Finally, it is noteworthy that glands of stilbestrol pellet-implanted rats have a Q_{O_2} of 5.3 ± 0.22 without added substrate and 4.6 ± 0.31 with added glucose. The corresponding figures for hypophysectomized rats given by Schuler (1941) are approximately 3.9 and 3.6. These differences may be related to the fact that, in the estrogenized animal, there is no evidence of a deficiency of thyrotrophic or adrenotrophic hormones (Nalbandov and Baum, 1948). The comparatively marked depression of testicular metabolism in the hypophysectomized rat may be an expression of deprivation of other trophic hormones as well as of gonadotrophins.

SUMMARY

The Q_{O_2} of rat testis does not change significantly over a wide age range if glucose is present in the medium. However, in the absence of added substrate, there is a progressive fall in Q_{O_2} with advancing age (Fig. 1).

Atrophy of the seminiferous tubules was produced by the technique of abdominal transplantation. The Q_{O_2} of the remaining tissue (which consisted principally of interstitial cells and Sertoli cells) was measured and found to be significantly higher than that of normal adult tissue in the absence of added substrate. The addition of glucose to the medium produced a lowering of the oxygen uptake (Fig. 3).

The glucose R.Q. of cryptorchid testis is 0.5 as compared with a value of 0.93 obtained with normal adult tissue (Fig. 5). The aerobic glycolysis of cryptorchid testis does not differ from that of normal adult glands (Fig. 6).

Androgen produces a lowering of the no-substrate Q_{O_2} in intact glands. The androgen effect is magnified in cryptorchid glands (Table 1).

The testes of stilbestrol-implanted rats have a higher Q_{O_2} than that previously reported for the glands of hypophysectomized rats (Table 2).

The implications of some of these findings are discussed.

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THE TEMPORARY NATURE OF THE INHIBITORY ACTION OF EXCESS IODIDE ON ORGANIC IODINE SYNTHESIS IN THE NORMAL THYROID¹.

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IN 1944, Morton *et al.* observed that when 300 mg. of sheep thyroid slices were incubated in 3 cc. of a bicarbonate-Ringer medium to which various amounts of I^{127} as inorganic iodide had been added, inhibition of thyroxine and diiodotryosine formation occurred when the added I^{127} exceeded 20 gamma. The resemblance of this *in vitro* effect to the action of excess iodide in Graves' disease in man led us to investigate this phenomenon in the normal intact thyroid (Wolff and Chaikoff, 1948a). In addition to confirming the inhibitory action of excess iodide upon thyroxine synthesis in intact thyroids, the investigation showed that such inhibition by a *single injection* of iodide was related to the level of plasma iodine (Wolff and Chaikoff, 1948b). So long as the concentration of plasma iodine exceeded 20-35 gamma per cent, organic binding of iodine failed to occur in the gland, and only when the concentration fell below this critical range did the gland resume its function of depositing iodine in an organic form. These observations in rats have been confirmed recently by Stanley (1948).

Evidence of an inhibitory action of excess iodide on thyroid function has also been obtained by a different approach. Purves and Griesbach (1946) have shown in rats that excess iodide (one mg. per day) potentiates the antithyroid action of a weak goitrogen. The administration of thiourea alone resulted in a slight degranulation of the acidophils of the anterior pituitary. But when large amounts of iodide were given along with the thiourea, the anterior pituitary presented a picture typical of complete thyroidectomy, i.e., complete degranulation of the acidophils. Mackenzie (1947) also has observed that the antithyroid effect of another weak goitrogen, sulfaguanidine, is augmented in rats fed a diet containing 500 gamma of iodine per gram.

It is well known that in the thyrotoxic patient, administration of excess iodide results in a return of the hyperplastic gland to a more

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normal state and in an increase in both total and thyroxine iodine of the gland (Gutman *et al.*, 1932). But in the normal rat, excess iodide, as judged by its ability to depress thyroxine formation in the thyroid, *in vitro* (Morton *et al.*, 1944) as well as *in vivo* (Wolff and Chaikoff, 1948b), appeared, rather, to resemble the action of anti-thyroid substances such as thiourea or its congeners. This resemblance between the effects of excess iodide and the antithyroid substances raised the question: does the maintenance of plasma iodide concentrations, well above 35 gamma per cent, for long periods induce histological changes in the thyroid and pituitary glands characteristic of a deficiency in circulating thyroid hormone? Although several attempts have already been made to study the effects of the administration of large amounts of iodide (Gray and Loeb, 1928; Weil, 1936; Hall, 1946), no reports have yet appeared in which the concentration of plasma iodide is shown to have been maintained at such high levels *without interruption* for as long as one month. Insurance against any interruption whatsoever is necessary because it was found that organic binding of iodide in the thyroid gland (i.e., hormone formation) is resumed when the plasma iodide falls below a critical level in the neighborhood of 20-35 gamma per cent.

A. THE EFFECTS OF INJECTIONS OF 500, 1,000 AND 2,000 GAMMA OF I^{127} , THREE TIMES DAILY FOR 1-4 WEEKS, UPON THE STRUCTURE OF THYROID AND PITUITARY GLANDS

Twenty-seven female rats of the Long-Evans strain, weighing from 166 to 232 grams, were injected intraperitoneally every eight hours for periods of one to four weeks with either 500, 1000, or 2,000 gamma of I^{127} as KI in 0.9 per cent saline (Table 1). All rats were sacrificed 10 hours after the last injection in order to insure adequate testing of blood iodine levels at a time which was two hours beyond the interval between successive injections. They were anesthetized with sodium pentobarbital. Blood was then removed from the heart.

TABLE 1. CONCENTRATIONS OF PLASMA TOTAL IODINE FOUND IN RATS INJECTED INTRAPERITONEALLY WITH IODIDE THREE TIMES DAILY

Amounts of iodide injected every 8 hours	Duration of injections	Plasma total iodine 10 hours after last injection
gamma	days	gamma per cent
500	1	156-205
500	2	137-175
500	3	165-394
500	5	125-269
500	14	555
500	21	225-425
1000	14	540-580
1000	21	265-375
2000	14	675-1060
2000	21	535-735

Total blood iodine was determined on two cc. samples of plasma, according to the method of Taurog and Chaikoff (1946).

After exsanguination, the thyroids were rapidly excised, weighed, and fixed in Bouin's. They were embedded in paraffin and prepared in multiple sections five micra thick. These were stained with hematoxylin and eosin. Liver, kidney, and heart were similarly treated.

Immediately after excision of the thyroids, the pituitary glands were removed, fixed in Zenker-Formol (9:1), embedded in paraffin, and prepared in sections 2-3 micra thick. They were stained by the method of Martins (1933) modified according to Griesbach (personal communication). The percentages of acidophils, basophils, and chromophobes were determined by a method similar to those employed Griesbach and Purves (1943) and by Floderus (1944). At least 2,000 cells were counted in each gland.

Table 1 lists the total plasma iodine values of animals killed at 1-5 days, and at two and three weeks after the start of the iodine injections. It will be seen that a minimum plasma iodine value of about 150 gamma per cent is established as early as 24 hours (i.e., after three injections of 500 gamma of iodine as KI), and is maintained at levels far in excess of that previously found necessary to prevent organic binding in the thyroid gland following single injections of 500 gamma of iodine.

Thyroid Histology (Figs. 1 and 2).—No evidence of hypersecretion of thyrotropic hormone was observed, i.e., epithelial hypertrophy or hyperplasia, or decrease in follicular colloid. On the contrary, reduction of follicular cell height and an increase in follicular colloid was a consistent finding in all animals that had received the iodide injections for one or more weeks.

Pituitary Cytology (Figs. 3 and 4).—Cytological examination of the anterior pituitary glands from these animals was undertaken as a more sensitive means of detecting a goitrogenic effect, if any, of a prolonged high plasma iodide level.

The sensitive response of this gland to very slight thyroxine deficiency has been clearly demonstrated in a series of studies by Griesbach and coworkers (1945, 1949) in thyroxine deficient rats. A decrease of 0.1-0.2 gamma in the amount of injected thyroxine necessary to maintain the anterior pituitary in a normal state can be detected by a significant increase in the percentage of the basophils. A more severe thyroxine deficiency (induced either by subtotal thyroidectomy or by the administration of a weak goitrogen) results in the appearance of large hyalinized basophils (thyroidectomy or signet ring cells) and some degranulation of the acidophils. Complete degranulation of the acidophils occurs only after total thyroidectomy or after prolonged treatment with a highly active goitrogen such as propylthiouracil. (fig. 4).

In Table 2, the anterior pituitary cell counts of the iodide-injected rats are compared with results obtained from the following types of

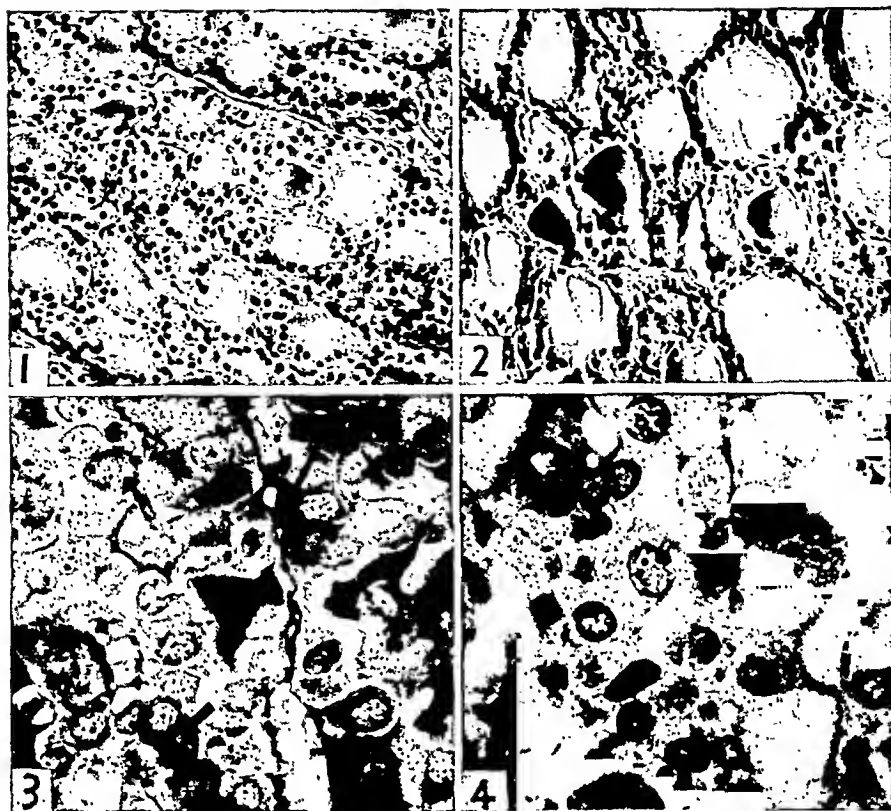


FIG. 1. Thyroid from control rat, maintained on diet containing 0.3 gamma iodide per gram. Note columnar epithelium and large vesicular nuclei. Hematoxylin and eosin; $\times 275$.

FIG. 2. Thyroid from rat injected with 2000 gamma iodide every eight hours for three weeks. Note flattened epithelium and smaller deeper staining nuclei. Hematoxylin and eosin; $\times 275$.

FIG. 3. Anterior pituitary from animal injected with 1000 gamma iodide every eight hours for three weeks. Arrows in upper portion of picture point to granulated acidophils which appear gray. Arrow at lower left points to a normal basophil. This does not differ from control pituitaries. Martins; $\times 750$.

FIG. 4. Anterior pituitary from rat maintained on diet of 0.2 per cent propylthiouracil for one month. The basophils contain large hyaline vacuoles (thyroidectomy cells) and constitute 20 per cent of the total number of cells. All other cells are chromophobes. Martins; $\times 750$.

rats: (1) controls, i.e., rats fed the same diet but not injected with iodide; (2) rats totally thyroidectomized; and (3) rats fed for one month a diet containing 0.2 per cent propylthiouracil.

The percentages of the various cell types found in the injected rats were well within the range found for control animals. When the anterior pituitaries of rats exhibiting thyroxine deficiency are compared with those of rats injected repeatedly with large amounts of iodide for periods up to four weeks, it becomes clear that the latter suffer from no deficiency in circulating thyroid hormone.

TABLE 2. DIFFERENTIAL CELL COUNTS OF ANTERIOR PITUITARIES OF RATS

Treatment	Anterior pituitary cell counts*			Remarks
	Acidophils†	Basophils	Chromophobes	
Control (untreated)	per cent of total 38-43	per cent of total 5-9	per cent of total 50-57	Cells of normal appearance.
500, 1000, 2000 gamma I^{127} thrice daily for 2 weeks	33-38	6-10	54-56	Cells of normal appearance.
500, 1000 2000 gamma I^{127} thrice daily for 3 weeks	35-41	6-10	48-57	Cells of normal appearance. See fig. 3.
500, 1000, 2000 gamma I^{127} thrice daily for 4 weeks	36-39	5-9	52-58	Cells of normal appearance.
One month after total thyroidectomy	0.7-1.1	21-29	70-78	Complete acidophilic degeneration. Majority of basophils of thyroidectomy type.
0.2 per cent propylthiouracil diet for one month	1.0-1.8	18-28	70-80	Complete acidophilic degeneration. Majority of basophils of thyroidectomy type. See fig. 4.

* At least 2000 cells were counted per gland.

† Degranulated acidophils were included in total per cent chromophobes.

B. FOR HOW LONG CAN THE THYROID GLAND BE INHIBITED WHEN A HIGH LEVEL OF PLASMA IODINE IS MAINTAINED?

It was shown in a previous report that the injection of a *single* dose of 500 gamma of iodide in the normal rat inhibited the formation of organic iodine for about 17 hours (Wolff and Chaikoff, 1948a). When the excretion of the iodide was prevented by nephrectomy, this effect of a *single* injection was prolonged to 32 hours (Wolff and Chaikoff, 1948c). But our failure to observe histological signs of thyroid deficiency in the preceding experiment led us to question the permanence of the inhibitory effect of excess iodide upon thyroxine formation in the gland. The following experiments were therefore designed to determine how long the normal rat thyroid can be inhibited by excess iodide.

A stock solution of KI, containing 500 gamma of I^{127} per cc. and an initial radioactivity of about 20 microcuries of I^{131} per cc., was prepared. One cc. of this *same* solution (i.e., 500 gamma I^{127}) was injected intraperitoneally into normal rats at eight-hour intervals for periods up to 111 hours. The levels of plasma total iodine⁴ established by this

⁴ Only a small fraction of this iodine is protein bound.

procedure (Table 3 and 4) are so high that dilution by the amount of iodine contributed by diet and breakdown of thyroxine is negligible. As a result, the specific activity of plasma remains the same as that of the injected dose throughout the experiment. Because of this, it was possible to calculate the amount of new organic iodine in the thyroid (Wolff and Chaikoff, 1948a).

Experiment 1—The rats used in this experiment weighed 150–222 gm. and, since weaning, had been fed a low iodine diet containing 0.3–0.4 gamma of iodine per gram. They had access *ad libitum* to this diet throughout the period of observation. The animals were sacrificed at 9, 18, 26, 50, 74, and 111 hours after the *first* injection and from 7 to 10 hours after the *last* injection. Blood was obtained from the heart, and the iodine content of plasma determined, as already described. The thyroids were excised and weighed. The organic and inorganic iodine fractions of the thyroids were separated, by means of trichloro-

TABLE 3. THE DURATION OF THE INHIBITORY ACTION OF EXCESS IODIDE IN THE THYROID GLAND

EXPERIMENT 1

(The rats were injected every 8 hours with 500 gamma of I^{127} -labeled I^{131})

Time after first injection	Plasma total iodine*	New† I^{127} in thyroid as:					
		Inorganic		Organic		Total	
hours		gamma	mg. per cent	gamma	mg. per cent	gamma	mg. per cent
9	68	2.9	11.2	0.18	0.69	3.1	11.9
	113	1.2	7.2	0.14	0.87	1.3	8.1
	100	4.0	13.8	0.11	0.38	4.1	14.2
	115	0.98	5.1	0.27	1.4	1.3	6.5
18	138	2.7	10.0	0.31	1.1	3.0	11.1
	183	1.9	11.9	0.42	2.6	2.3	14.5
	185	2.4	12.0	0.38	1.9	2.8	13.9
	140	2.5	11.6	0.30	1.4	2.8	13.0
26	145	2.2	11.4	0.38	2.0	2.5	13.4
	83	0.92	4.6	0.29	1.4	1.2	6.0
	105	2.7	10.4	1.1	4.2	3.8	14.6
	88	3.9	18.1	1.0	6.3	4.9	24.4
50	170	2.3	10.0	2.9	12.6	5.2	22.6
	170	0.89	4.4	5.1	25.5	6.0	29.9
	345	3.2	16.6	1.1	5.8	4.3	22.4
	153	1.7	6.7	2.0	8.0	3.7	14.7
74	252	3.0	15.0	3.4	17.0	6.4	32.0
	302	3.3	13.2	2.6	10.4	5.9	23.6
	178	1.3	7.6	4.6	27.0	5.9	34.6
	240	1.4	6.5	5.8	27.6	7.2	34.1
111	172	1.6	5.6	10.7	38.2	12.3	43.8
	190	1.6	6.7	4.6	20.0	6.2	26.7
	78	0.65	3.8	7.0	41.1	7.7	44.9
	300	0.83	4.0	11.4	54.2	12.2	58.2

* Only a small fraction of this iodine is protein-bound.

† Refers to I^{127} as calculated from the radioactivity recovered in the gland.

acetic acid, as described in an earlier report.⁵ The results of this experiment are recorded in Table 3.

It was shown that when the gland escaped from the inhibitory effects of a single injection of excess iodide, the amount of *new* I^{127} organically bound in the thyroid gland was always well in excess of one gamma (see fig. 6 of Wolff and Chaikoff, 1948a). During inhibition, on the other hand the amounts of *new* I^{127} so bound were below this amount. Hence for the present study, we have considered the gland to have escaped from inhibition when the total amount of *new* I^{127} bound organically exceeded one gamma.

In experiment 1 (Table 3), inhibition occurred during the earlier intervals, after the establishment of a high plasma iodine level. In two of the rats, more than one gamma of *new* organic iodine had accumulated in the thyroids 26 hours after the start of the injections. By the time 50 hours had elapsed, the gland showed a renewed capacity to bind iodine organically—*this occurred while the plasma iodine was still high*. At later intervals, more and more of the *new* iodine was organic.

Experiment 2—The rats weighed 210–272 gm. and had been raised on the same low-iodine diet as that used for experiment 1. They were injected every eight hours with 500 gamma of I^{131} labeled iodide, prepared as described in experiment 1, above. All rats were sacrificed two hours after the last injection. For this reason, plasma iodine values, as well as the inorganic iodine content of the thyroid glands, are higher than those observed in experiment 1. These results are shown in Table 4.

Up to 26 hours, the amounts of the injected I^{127} organically bound in the thyroid did not exceed one gamma, the value arbitrarily set as the escape level. At 34 hours, the thyroids of two of the rats still showed inhibition whereas in the other three rats examined at this interval, about 1.3 gamma of *new* I^{127} were organically bound.

Inhibition was still observed in two of the four rats examined at 42 hours and in one of the five rats examined at 50 hours. But 58 hours

⁵ It has already been shown (Wolff and Chaikoff, 1948a) that no inorganic iodide appears in the *insoluble* fraction. The nature of the trichloroacetic acid-soluble fraction was tested here by a method similar to that described by Taurog *et al.* (1947). Rats were injected with 500 gamma of I^{127} containing I^{131} and were killed five or 22 hours thereafter. Glands of three rats were pooled and ground with one cc. of cold 10 per cent trichloroacetic acid. The resulting precipitate was washed twice with 2.5 cc. of 5 per cent trichloroacetic acid and discarded.

To the combined supernatants (the trichloroacetic acid-soluble fraction) were added 0.1 cc. of a 2N KI solution, as carrier, and 0.5 cc. of 0.1 N KIO_3 . The I_2 so formed was then extracted three times with equal volumes of CCl_4 . The combined CCl_4 -extracts were then re-extracted three times with dilute (0.1 N) sodium thiosulfate. The radioactivity of the trichloroacetic acid-residue, the CCl_4 -fraction, and the thiosulfate fraction was measured. Ninety-five per cent of the iodine originally present in the trichloroacetic acid-soluble fraction was recovered in the thiosulfate solution. This finding indicates that the iodine in this fraction must have been either in the form of I^- or I_2 . *This was the case in the early intervals while the excess iodide exerted its inhibitory action as well as later when escape from inhibition had occurred in the gland.*

TABLE 4. THE DURATION OF THE INHIBITORY ACTION OF EXCESS
IODIDE IN THE THYROID GLAND
EXPERIMENT 2

(The rats were injected every 8 hours with 500 gamma of I^{131} -labeled I^{127})

Hours after first injection	Plasma total iodine*	New† I^{127} in thyroid as:					
		Inorganic		Organic		Total	
hours	gamma per cent	gamma	mg. per cent	gamma	mg. per cent	gamma	mg. per cent
10	440	9.0	45.0	0.42	2.1	9.4	47.1
10	275	8.5	35.4	0.24	1.0	8.7	44.1
10	260	9.0	36.0	0.28	1.1	9.3	37.1
10	240	7.5	31.2	0.14	0.58	7.6	31.8
18	436	11.0	52.3	0.49	2.3	11.5	54.6
18	366	8.5	42.5	0.34	1.7	8.8	44.2
18	410	8.0	36.4	0.80	3.6	8.8	40.0
18	320	9.5	36.5	0.37	1.4	9.9	37.9
18	470	10.0	40.0	0.26	1.0	10.3	41.0
26	340	9.0	40.8	0.70	3.2	9.7	44.0
26	240	5.0	26.3	1.0	5.3	6.0	31.6
26	290	7.5	28.8	0.50	1.9	8.0	30.7
26	240	6.5	20.9	0.70	2.3	7.2	23.2
34	475	6.5	28.2	1.2	5.2	7.7	33.4
34	670	6.5	29.5	0.35	1.6	6.9	31.1
34	360	5.0	23.8	0.75	3.6	5.8	27.4
34	225	5.5	19.0	1.5	5.2	7.0	24.2
34	430	8.5	27.4	1.3	4.1	9.8	31.5
42	400	7.0	29.1	0.32	1.3	7.3	30.4
42	260	3.9	21.9	0.65	3.8	4.6	25.7
42	475	4.4	17.6	2.3	9.2	6.7	26.8
42	270	8.0	38.1	1.4	6.7	9.4	44.8
50	400	2.8	14.7	0.39	2.1	3.2	16.8
50	390	4.8	21.8	3.2	14.5	8.0	36.3
50	385	4.5	18.7	2.5	10.4	7.0	29.1
50	340	4.2	16.8	2.5	10.0	6.7	26.8
50	340	4.2	18.2	2.3	10.0	6.5	28.2
58	295	2.8	11.7	4.5	18.7	7.3	30.4
58	250	5.5	21.1	2.1	8.1	7.6	29.2
58	400	4.4	22.0	3.9	19.5	8.3	41.5
58	230	2.7	15.0	1.5	8.3	4.2	23.3
58	230	3.9	15.6	4.0	16.0	7.9	31.6

* Only a small fraction of this iodine is protein-bound.

† Refers to I^{127} as calculated from the radioactivity recovered in the gland.

after the first injection, escape occurred in all rats; their thyroids contained 2.3–3.2 gamma of new organically bound iodine.

DISCUSSION

The inhibitory action of excess iodide on organic-iodine synthesis in the thyroid gland has been confirmed here but shown to be temporary in nature. The maximum duration of this effect was 50 hours. Escape from inhibition was detected as early as 26 hours, even though plasma total iodine was maintained at levels at which inhibition was initially established. Since it is probably the inorganic iodine within the gland that is the immediate agent responsible for the inhibitory

effect,⁶ it became pertinent to determine whether escape from inhibition after 26 hours occurred because the gland lost its capacity to hold high concentrations of inorganic iodide. Tables 3 and 4 show, however, that when escape occurred, the concentrations of inorganic iodide in the gland were as high as those which, in the earlier intervals, caused inhibition.

TABLE 5. REACCUMULATION OF ORGANIC IODIDE IN THYROIDS OF RATS TREATED WITH EXCESS IODIDE

(All rats were fed for the first 19 days a diet containing 0.2 per cent propylthiouracil. At the end of this period the first three rats were sacrificed. The remainder were fed a normal diet for nine or 15 days. Half of the animals of each group received by intraperitoneal injection 500 gamma of iodide as KI thrice daily.)

Time after propylthiouracil	I^{127} injected every 8 hours	Thyroid iodine			
		Inorganic	Organic		Total
			Non-thyroxine	Thyroxine	
days*	gamma	gamma	gamma	gamma	gamma
0	0		0.11†	0.22	0.33
0	0		0.14	0.16	0.30
0	0		0.11	0.27	0.38
9	0		0.81	0.17	0.98
9	0		0.63	0.22	0.85
9	0		2.2	0.54	2.7
9	500	5.4	4.8	0.71	10.9
9	500	7.2	1.8	0.40	9.4
9	500	3.0	4.0	1.0	8.0
15	0		2.2	0.45	2.7
15	0		4.3	0.83	5.1
15	0		2.6	0.70	3.3
15	500	4.0	3.8	0.75	8.6
15	500	5.0	3.6	0.45	9.1
15	500	1.2	4.6	1.1	6.9

* Normal rats of this group contained about 1.5 gamma of thyroxine and 4.2 gamma of nonthyroxine iodine.

† In the rats that received no iodide injections the inorganic iodine was not separated from the nonthyroxine fraction.

Although the problem of exchange has been discussed elsewhere (Chaikoff and Taurog, 1948), the fact that we are dealing here with higher concentrations of iodide leads us to reconsider it. It might be argued that prolonged exposure of the gland to high levels of inorganic iodide results in an exchange between inorganic I^{131} and organically bound I^{127} . If this were the case, the recovery of the I^{131} in the organic fraction (Tables 3 and 4) would not be indicative of renewed synthesis or escape. To test this possibility it was necessary to measure the accumulation of organic iodine in the thyroid gland without resorting to the use of I^{131} . The thyroids of rats were first depleted of their iodine stores by feeding 0.2 per cent propylthiouracil in the diet for 19 days. Thereafter, they were fed a normal diet and injected with excess iodide

⁶ Cf. *in vitro* experiments by Morton *et al.* (1944).

(500 gamma every eight hours) for 15 days. As shown in Table 5, reaccumulation of organic I^{127} was not prevented by the excess iodide. In addition to confirming the temporary nature of the inhibitory action of excess iodide upon organic binding of iodine by the thyroid gland, these results lead us to conclude that exchange reactions could not account for the increase of organically bound I^{131} shown in Tables 3 and 4.

In view of the temporary nature of iodide inhibition in the normal rat thyroid, we must reopen the question as to whether a block in the formation of organic iodine is an adequate explanation for the characteristic action of excess iodine (Lugol's) in Graves' disease. An answer to this question must now await investigations in the human thyroid. The recent discovery by Stanley and Astwood (1948) that KSCN expels inorganic iodide from the thyroid offers, for the first time, a simple approach to this problem in man without surgical interference. In patients that have been injected with I^{131} , the inorganic iodide can be discharged from the thyroid gland by thiocyanate, and the organic I^{131} measured by external counting.

SUMMARY

Experiments designed to test the duration of inhibition, by excess iodide, on organic binding of iodine in the thyroid gland are described.

It is shown that the inhibitory action of excess iodide on the normal rat thyroid is temporary in nature. Inhibition lasted for about 26 hours. Despite the continued maintenance of a high level of plasma total iodine (100–200 gamma per cent), the formation of significant amounts of organic iodine was resumed after 26 hours.

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THE RELATIONSHIP OF THE MOUSE ADRENAL CORTEX TO THE PITUITARY

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The course of events in the mouse adrenal cortex after hypophysectomy has been dealt with (Jones, 1949; 1950). It seems that the zona glomerulosa is independent of the pituitary in so far as it may be considered a histological entity, and that both the zona fasciculata and the X zone degenerate after removal of the hypophysis. In this paper the results of the injections of different, albeit impure, fractions of the anterior pituitary into hypophysectomized mice are given.

The mice chosen are those whose adrenals possess an X zone and these fall into various categories. In the virgin female the X zone is a distinctive layer of cells surrounding the medulla and it persists for a varying length of time in adult life (Howard, 1927; Deanesly, 1928). In the male the X zone normally disappears at puberty. If, however, the immature mouse is castrated the X zone continues to develop and comes to occupy a large portion of the cortex as in the virgin female. The adult male mouse has no X zone, but postpuberal castration allows a new zone to arise around the medulla. This new cortical zone is very similar, histologically, to the X zone and it is termed the "secondary" X zone (Howard, 1939). These three categories of mice are considered, then, and the relationship of the various cortical zones to the pituitary is explored.

One injected substance is employed which is not of pituitary origin, namely, human chorionic gonadotrophin. This is used, firstly, because it has some properties in common with the luteinizing hormone of pituitary origin, and secondly, because it is a hormone of pregnancy. In the female mouse the X zone disappears during first pregnancy, and the factors effecting this are unknown. Although the hormone produced from the rodent placenta is not the same type as that from man (Astwood and Greep, 1938), nevertheless it was considered that the results from the use of human chorionic gonadotrophin might throw some light on the disappearance of the X zone in the primiparous female mouse.

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TABLE 1. TEST OF GONADOTROPIN PREPARATIONS USING HYPOPHYSECTOMIZED, IMMATURE MALE ANIMALS, FOUR IN EACH GROUP

	Body wt.	Testes	Seminal vesicles	Ventral prostate
(a) Mice				
Controls	14.3	79.6	6.1	1
Gonadotrophin I	12.8	100.6	24.1	4.3
(b) Rats				
Controls	43.2	140	9.5	7.4
Gonadotrophin I	38.2	391	18.7	35.9
Gonadotrophin II	44.9	368	17.0	25.7
Gonadotrophin III	43.5	225	11.2	24.1

MATERIAL AND METHODS

The general plan of the experiment was to take the various categories of mice which possess an X zone and after removal of the pituitary, to inject adrenocorticotrophin and gonadotrophins of several types, followed by examination of the adrenal glands. The spayed female was used in some cases in order to obviate possible hormonal mediation via the ovaries.

The mice, which were "Swiss" albino, were maintained on Purina Laboratory Chow or on a synthetic diet, with tap water *ad libitum*. Diet made no detectable difference to adrenal reactions. The advantage of the synthetic diet was that with its use the survival rate of animals after hypophysectomy is very good. The diet was made up from Shaw (1947) and a detailed analysis of its use is given in Shaw and Greep (1949) and see also Jones (1949).

The mice were killed with chloroform. The organs listed in Tables 1, 2 and 3 were dissected out and weighed on a Roller-Smith balance. The right adrenal and ovaries were fixed in Bouin's fluid, embedded in paraffin wax, cut at 7 μ , and stained in Harris' hematoxylin and eosin or in Heidenhain's Azan. The left adrenal was fixed in 10 per cent neutral formalin and sectioned

TABLE 2. BODY AND ORGAN WEIGHTS OF VIRGIN FEMALE MICE IN THE DIFFERENT EXPERIMENTAL CATEGORIES

Experimental category	No. in group	Body wt.	Adrenals	Ovaries	Uterus	Thymus
1. Intact controls	20	19.7	6.8	5.6	44.1	60
2. Hyp. controls	20	18.7	3.0	2.5	8.2	54
3. Hyp. & Gon. I	7	19.2	—*	4.4	19.3	83
4. Spayed, hyp. & Gon. I	5	17.7	4.1	—	11.4	64
5. Spayed & Gon. I	5	22.6	8.3	—	12.9	71
6. Hyp. & Gen. II	5	18.2	—*	7.0	42.4	82
7. Spayed & Gon. IV	5	18.7	6.4	—	12.5	58
8. Spayed, hyp. & Gon. IV	5	17.2	3.5	—	11.9	74
9. Hyp. & Gon. IV	6	19.1	—*	4.4	17.5	72
10. Spayed, hyp., Gon. IV & estrogen	4	20.6	2.9	—	49.0	75
11. Spayed, hyp., Gon. IV & progesterone	2	18.7	3.7	—	31.7	33
12. Intact & ACTH	7	16.9	—*	3.8	28.7	20
13. Hyp. & ACTH	12	14.9	5.31	2.4	12.4	23

* Weights not taken

Abbrev.: Hyp. = hypophysectomized

Gon. = gonadotrophin preparation

at 15μ on the freezing microtome. These sections were treated routinely in the following manner: (1) Stained with Sudan IV; (2) stained with Sudan IV and counterstained with hematoxylin; (3) stained with Sudan black; (4) stained with Schiff's reagent; (5) mounted in glycerine and examined with the polarizing microscope and with the fluorescence microscope; (6) extracted with acetone for $\frac{1}{2}$ hour at room temperature and examined as in (5), above; and (7) treated by the Schultz method for cholesterol.

The interpretation of the use of these histochemical methods in the mouse is discussed by Jones (1949; 1950). Suffice to say here that they are employed as indicative of acetone-soluble substances which seem to vary in expression with the activity of the adrenal cortex.

TABLE 3. THE ORGAN WEIGHTS OF POST-PUBERALLY CASTRATED MALE MICE, VARIOUSLY TREATED, COMPARED TO THOSE OF NORMAL INTACT MALES

	No.	Body wt.	Adrenals	Seminal vesicles	Thymus
Castrated	8	30.7	6.6	10.3	68
Castrated & hyp.	5	23.8	2.9	9.0	42
Castrated, hyp. & Gon. I or II	6	25.8	3.9	8.9	43
Intact normals	7	30.9	4.4	177.0	69

On the group with a secondary X zone in the adrenal, some further methods were employed. Reducing substances in the adrenal cortex were located by the method of Deane and Morse (1948). These reducing substances may well be mainly ascorbic acid. The method for the histochemical identification of alkaline phosphatase was that of Gomori (1939), using sodium β glycerophosphate as a substrate. Values for "total" and "free" cholesterol in the adrenals were obtained by use of the modified Schoenheimer-Sperry method (Sperry, 1938).

The operation of hypophysectomy was performed by the parapharyngeal approach under "nembutal" anesthesia. The survival was good. The completeness of hypophysectomy was judged by the examination of serial sections of the appropriate area in several cases. In the remainder, the success of the operation was established by examination of the sella turcica area under the binocular microscope ($\times 15$). Added confirmation was given, where relevant, by the organ weights and by the histological condition of the ovaries and adrenals. The term "hypophysectomy" is employed in this paper to mean that the anterior and intermediate lobes are removed (Newton and Richardson, 1940). Although the posterior lobe as such is removed, components above the diaphragma sella may substitute for it physiologically.

The preparations used in these experiments were as follows:

1) Gonadotrophin I. This was prepared from sheep pituitaries by Armour & Co., Lot FW-234. Besides gonadotrophin it contains, according to the makers: prolactin, 0.16 unit per mg.; thyrotrophin, 0.4 unit per mg.; growth, 0.2 Evans unit per mg. (epiphyseal disc method); adrenocorticotrophin, negligible; Posterior lobe, oxytocic (rooster), 0.2 unit per mg. and pressor (cat), 0.2 unit per mg.

The preparation was tested for its gonadotrophic properties by injection into hypophysectomized immature male rats and mice of 21-23 days of age

(see Table 1). The assumption is made that luteinizing hormone (LH; ICSH) stimulates the interstitial cells of the testis to produce androgens without causing sperm formation which is considered to be the function of the follicle stimulating hormone (FSH) (Greep, van Dyke and Chow, 1942). The ventral prostate in the rat is the most sensitive indicator of androgen activity, the seminal vesicles being less responsive; in the mouse they seem equally sensitive. From Table 1 it will be seen that the preparation is rich in LH but it is not pure in this respect. The increase in weight of the testes indicates that FSH is also present. The effect of this preparation on the hypophy-

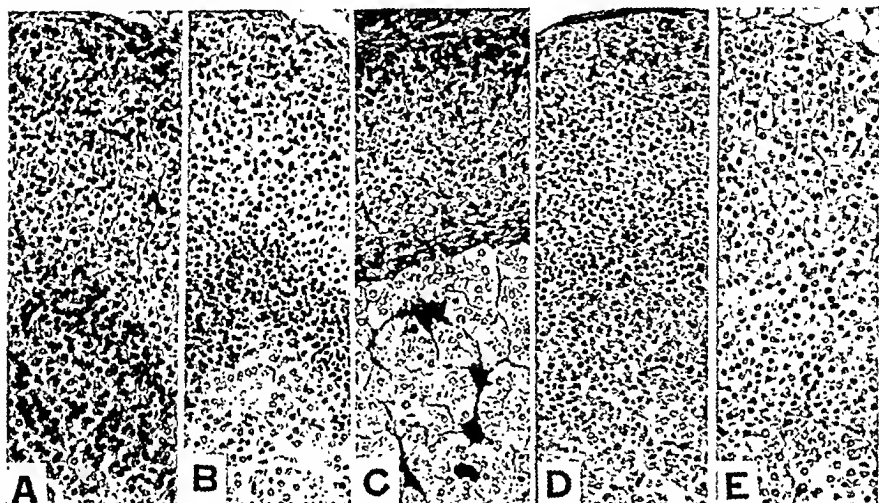


FIG. 1. Sections of adrenals of male mice. Bouin; Hemotoxylin and eosin except 1c which is stained with Heidenhains Azan.

- (a) Normal immature male, 25 days old, showing the prominent X zone.
- (b) Immature male, hypophysectomized for 5 days. The zona glomerulosa has a normal appearance, the zona fasciculata and the X zone are degenerating.
- (c) Immature male, hypophysectomized for 5 days, injected with Gonadotrophin I for 4 days. The LH has caused the production of androgens from the testes which in turn have destroyed the X zone. A medullary connective tissue capsule has appeared.
- (d) Castrated immature male, hypophysectomized for 5 days, and injected with Gonadotrophin I for 4 days. In this case no androgens are produced in the absence of the testes and the LH maintains the X zone in a condition approaching that of the normal.
- (e) Young adult male. The androgens produced concomittant with puberty have destroyed the X zone, and a medullary connective tissue capsule is present. This effect was achieved in (c), above, in the hypophysectomized animal using exogenous LH.

sectomized virgin female also indicated that some FSH was present, although LH activity was clearly dominant. The weight of the ovaries was nearly doubled as against the hypophysectomized controls, and the uterus weight was more than doubled (Table 2). Histologically the ovaries showed marked corpora lutea formation, whereas those of the hypophysectomized controls showed only small follicles.

The condition of the adrenals of the male mice used in the test of this

onto the X zone without the intervention of a zona reticularis. There is no medullary connective capsule. The X zone is an obvious layer of cells when seen in sections stained in hematoxylin and eosin preparations after Bouin's fluid. The cytoplasm is very eosinophil, the nuclei large, round and basophilic. At these ages the X zone is well developed and shows none of the spontaneous "fatty" degeneration which occurs later in life (Howard, 1927). In the sections prepared after the histochemical methods, the zona glomerulosa and zona fasciculata are sudanophilic (fig. 6), Schiff positive (fig. 10), give the Shultz cholesterol reaction, have a stable greenish-blue fluorescence in ultraviolet light, and contain birefringent particles (fig. 14). The cytoplasmic droplets produce these reactions except perhaps in the case of the birefringent particles (Yoffey and Baxter, 1947), although other observations (Deane and Jones, unpublished) would indicate that the birefringence originates in these droplets for the most part. Positive reactivity is removed by acetone extraction. The overall picture shows that the zona glomerulosa in the Swiss albino is less reactive than the zona fasciculata, that is, the cytoplasmic droplets are less numerous in the zona glomerulosa. The X zone does not contain substances which react with the Sudans, or the Schiff reagent or give a positive reaction with the Schultz test. No birefringent particles are formed in the zone with or without formalin fixation. The fluorescence is a nonspecific bluish-black common to many tissues of the body. The medulla, which does not concern this paper, is not reactive to any of these tests.

The hypophysectomized virgin female. After removal of the pituitary, both the zona fasciculata and the X zone degenerate (Jones, 1949; 1950). Twelve to 14 days after operation the nuclei of the zona fasciculata have become pyknotic and the cell cytoplasm has shrunk (fig. 2). The cytoplasmic droplets have coarsened and coalesce in parts

FIG. 1. Normal virgin female mouse, age 55 days. The juxtamedullary X zone stands out as a wide deeply stained layer, contrasting with the rest of the cortex.

FIG. 2. Virgin, hypophysectomized at 40 days of age. Length of time hypophysectomized, 14 days. The zona glomerulosa is not markedly different from that of the control. The degeneration of the zona fasciculata is well advanced. Areas of degeneration are particularly apparent in the region of the inner zona fasciculata and the outer part of the X zone. The X zone has collapsed and is represented by a layer of pyknotic nuclei lying against the medulla.

FIG. 3. Virgin, spayed at 43 days, hypophysectomized at 45 days. Length of time hypophysectomized, 14 days. Injections of Gonadotrophin I for 12 days. The X zone is maintained with the nuclei normal in appearance and the cell cytoplasm staining with eosin. The zona glomerulosa is normal in appearance. The zona fasciculata is degenerating and narrow.

FIG. 4. Virgin, hypophysectomized for 14 days. Injection of adrenocorticotrophin for 12 days. The zona fasciculata is wide and well maintained. The X zone is collapsed, its nuclei pyknotic, the cell cytoplasm shrunk and non-eosinophilic. The zona glomerulosa has a normal appearance.

FIG. 5. Virgin, hypophysectomized for 12 days. Human chorionic gonadotrophin for 10 days. The X zone has disappeared and a medullary connective tissue capsule is present. There is a tendency towards hyperemia.

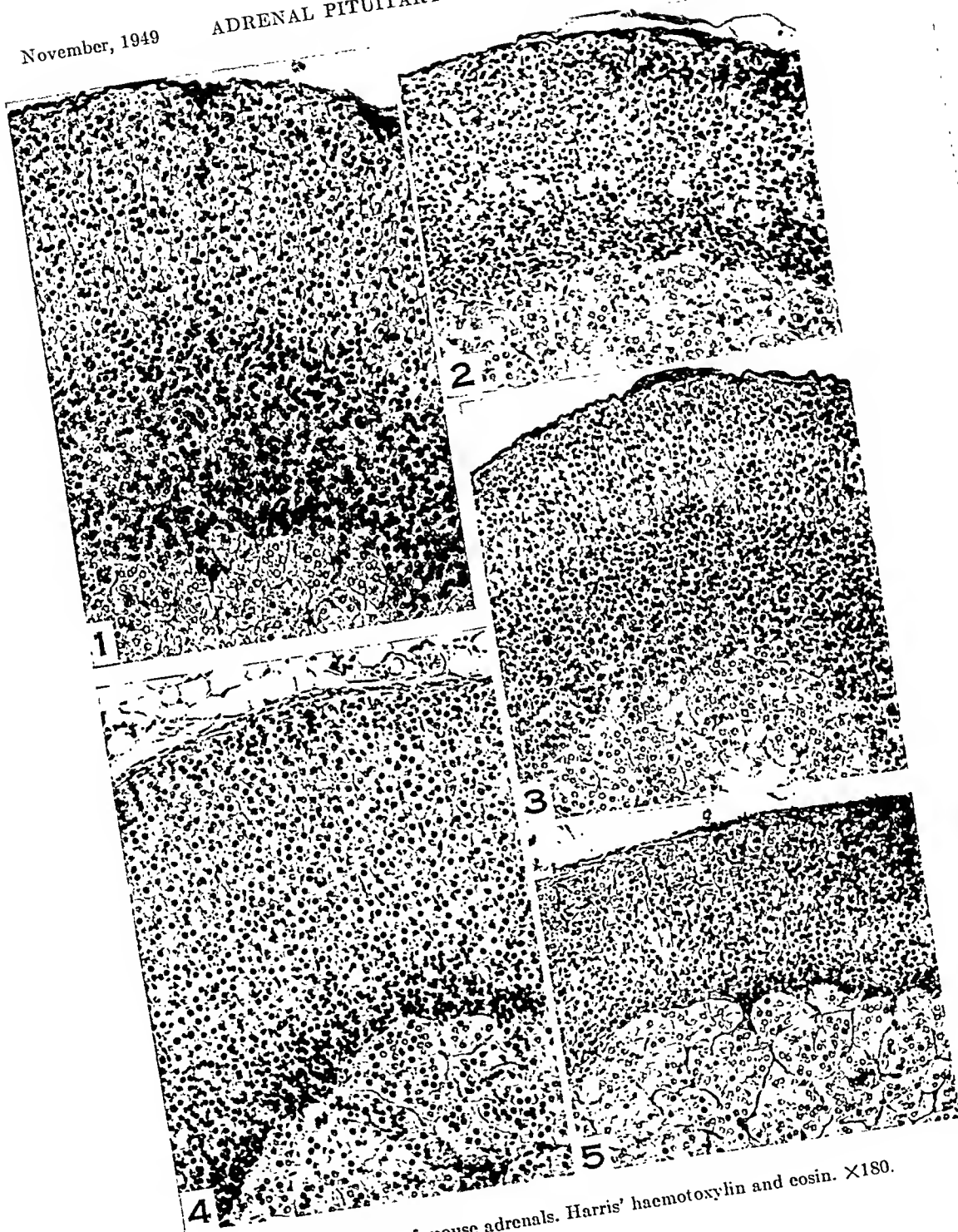


PLATE I. Sections of mouse adrenals. Harris' haematoxylin and eosin. $\times 180$.

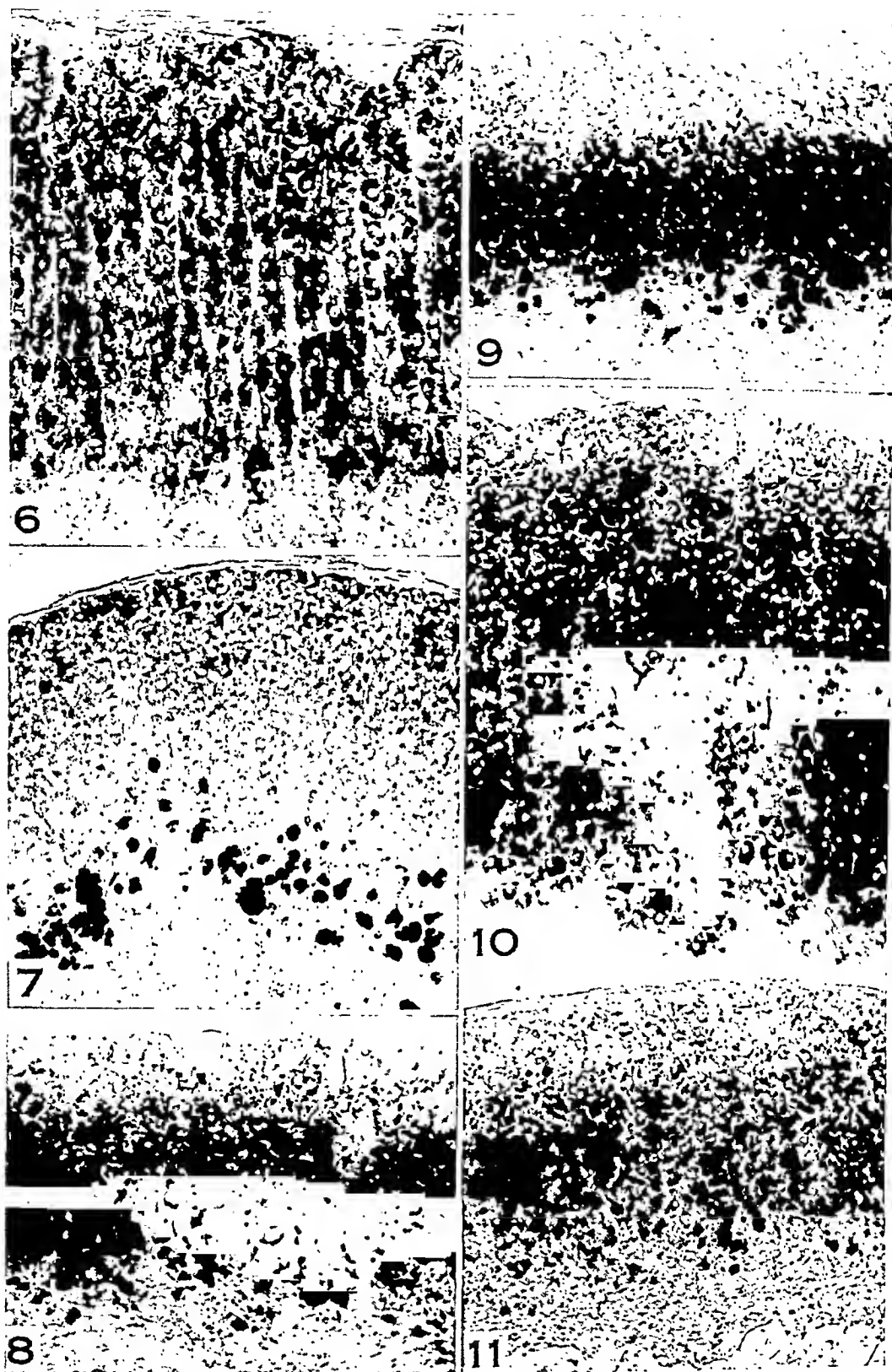


PLATE II. Sections of the adrenals of virgin female mice cut on freezing microtome at 15 μ . Figures 6-9 stained with Sudan IV. Figures 10 and 11 with the Schiff reagent. $\times 180$

especially toward the inner region of the zona fasciculata where actual lobules of sudanophilic material appear. The staining with Sudan is more intense (fig. 7), and the Schiff reagent (fig. 11) gives a deeper color when compared to that in the normal animal. The greenish-blue fluorescence has vanished and only the nonspecific blue-black remains. The birefringent particles have become larger, and there is a tendency to clump together (fig. 15). The X zone has lessened in area, the nuclei have become pyknotic, the cell cytoplasm considerably reduced and the zone is no longer eosinophilic (fig. 2). The X zone is still not reactive to the histochemical tests, except that sudanophilic degenerative areas may have appeared, especially in the outer part (fig. 7). The changes in the adrenal are no different when the animal is spayed 48 hours before removal of the pituitary. The zona glomerulosa, on the other hand, does not degenerate after hypophysectomy and, in the histological preparations, differs little in appearance from that of the normal. The zone, however, by 14 days after operation, shows little to no sudanophilic, Schiff positive, and Schultz positive material; birefringent particles are rare and it shows only the blue-black fluorescence.

The hypophysectomized virgin given injections of Gonadotrophin I. After injections of Gonadotrophin I for 12 days, the adrenal presents a different appearance. In histological preparations both the zona glomerulosa and the zona fasciculata are much the same as in the untreated hypophysectomized animal (fig. 3). The X zone does not show the typical degeneration, but the cells are well maintained, the nuclei round, the cytoplasm plentiful and eosinophilic. There are no degenerative areas to be found therein. The zone itself is wide

FIG. 6. Normal, aged 60 days. The sudanophilia of the zona glomerulosa and the zona fasciculata is apparent, and is confined to the cytoplasmic droplets. Only a portion of the X zone is visible, at the bottom of the photograph. It is not sudanophilic. The medulla is not shown.

FIG. 7. Virgin, hypophysectomized for 14 days. Adrenocorticotrophin for 12 days. The sudanophilia of the zona glomerulosa and the outer region of the zona fasciculata may be seen and this lessens toward the inner zona fasciculata. Lobules of degeneration can be seen in the inner region of the zona fasciculata and in the X zone.

FIG. 8. Virgin female hypophysectomized for 14 days. Much of the sudanophilia has disappeared from the zona glomerulosa. The zona fasciculata is densely stained, and the droplets have coarsened and coalesced. A few sudanophilic lobules can be seen in the X zone at the bottom of the photograph.

FIG. 9. Virgin hypophysectomized for 14 days. Gonadotrophin I for 12 days. The absence of sudanophilic material from the zona glomerulosa is particularly marked. The zona fasciculata presents the typical degenerated appearance after hypophysectomy. The X zone in these injected animals is well maintained. This is not apparent in the illustration as the zone is not sudanophilic.

FIG. 10. Normal virgin. The zona fasciculata has reacted strongly with the Schiff reagent, the zona glomerulosa less so. The X zone does not react, a small portion is to be seen at the bottom of the photograph.

FIG. 11. Virgin, hypophysectomized for 14 days. The zona glomerulosa reacts weakly. The degenerating zona fasciculata shows varying strength of reaction. The degenerating X zone is now reactive.

and approaches that of the normal (cf. fig. 3). The pictures after Sudan (fig. 9), Schiff (fig. 12), the fluorescence and birefringence (fig. 16) are similar to that of the untreated hypophysectomized animal. The maintained X zone is not reactive to the histochemical tests used, following the zone of the normal adrenal in this respect.

The spayed, hypophysectomized female injected with Gonadotrophin I. Spayed, hypophysectomized females injected with this preparation possessed a well-maintained X zone, approaching that of the normal in histological appearance. The undegenerated zona glomerulosa, the regressing zona fasciculata and the X zone of normal appearance can be clearly seen in the illustration (fig. 3). There is no significant difference between the hypophysectomized animal injected with Gonadotrophin I and the spayed with similar treatment. This seems to indicate that the action of the Gonadotrophin is a direct one on the X zone, without ovarian mediation. Illustrations in figures 9, 12 and 16 serve to represent both categories.

The spayed female (not hypophysectomized) injected with Gonadotrophin I. No histological or histochemical difference could be detected between the adrenals of these animals and those of the normal mice. There is an indication in the treated animals that the X zone is of larger area. This is an impression and the numbers are too small for statistical confirmation.

The hypophysectomized female injected with Gonadotrophin II. After this treatment, the zona glomerulosa and the zona fasciculata were similar to those of the untreated hypophysectomized controls. On the other hand, the degeneration of the X zone had been fairly

FIG. 12. Virgin, hypophysectomized for 14 days, Gonadotrophin I for 12 days. Little reaction in the zona glomerulosa, moderate in the zona fasciculata, with some degenerative areas giving a strong reaction. The X zone, which is well maintained in these animals, does not react, but the faint cytoplasmic wash brings it out in the photograph. The medulla lies to the bottom of the photograph.

FIG. 13. Virgin, hypophysectomized for 14 days. Adrenocorticotrophin for 12 days. The zona glomerulosa and the outer region of the zona fasciculata react strongly, the inner zona fasciculata less so. The narrow collapsed X zone is non-reactive.

FIG. 14. Normal virgin. A mixture of fine and coarse birefringent particles are scattered in the zona glomerulosa and the zona fasciculata, although particles are less prevalent in the zona glomerulosa. The X zone does not show as it does not contain birefringent material.

FIG. 15. Virgin, hypophysectomized for 14 days. The outer connective tissue capsule shows in part due to its intrinsic birefringence as do some of the radial connective tissue fibers of the cortex. The zona glomerulosa has few birefringent particles in it. The zona fasciculata possesses mainly coarse clumps of birefringent material.

FIG. 16. Virgin, hypophysectomized for 14 days. Gonadotrophin I for 12 days. The picture is similar to 15 above, indicating that the gonadotrophin had no effect on the zona glomerulosa and the zona fasciculata. The non-degenerated X zone does not show as it contains no birefringent particles.

FIG. 17. Virgin, hypophysectomized for 14 days, adrenocorticotrophin for 12 days. A mixture of fine and coarse birefringent particles are present in the zona glomerulosa and in the outer zona fasciculata. The inner zona fasciculata contains few such particles. The degenerative lobules in the inner part of the zona fasciculata and the X zone show a faint fine birefringence.

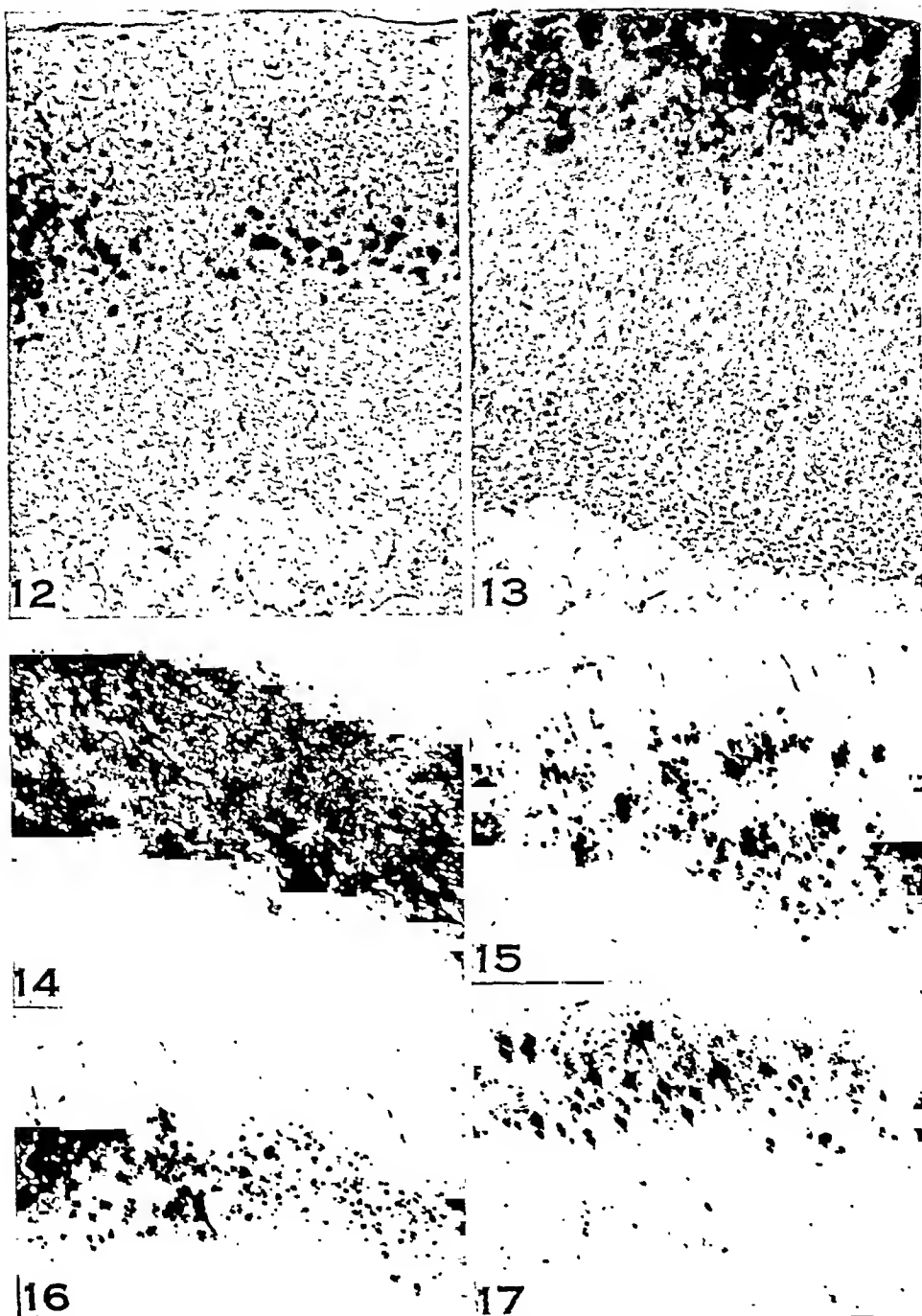


PLATE III. Sections of adrenal of virgin female mice, cut on the freezing microtome at $15\ \mu$. Figures 12 and 13 after the Schiff reagent. $\times 180$. Figures 14-17 mounted plain in glycerine, and viewed with the polarizing microscope. $\times 120$.

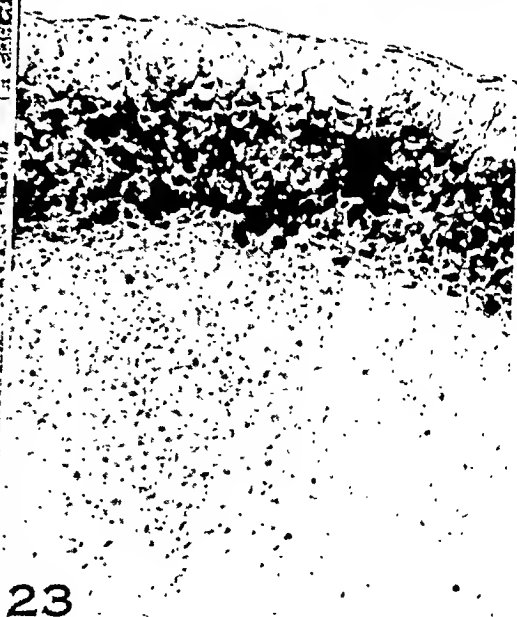
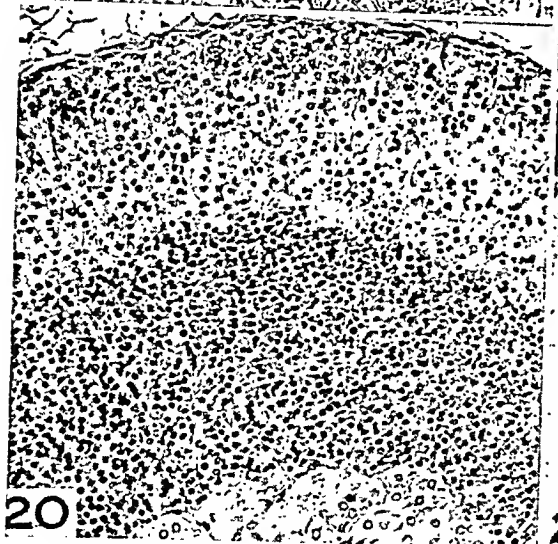
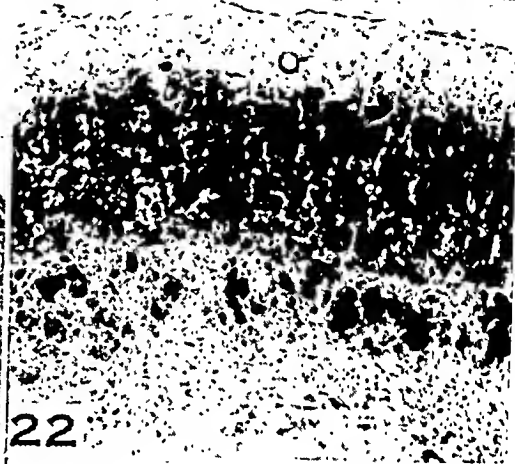
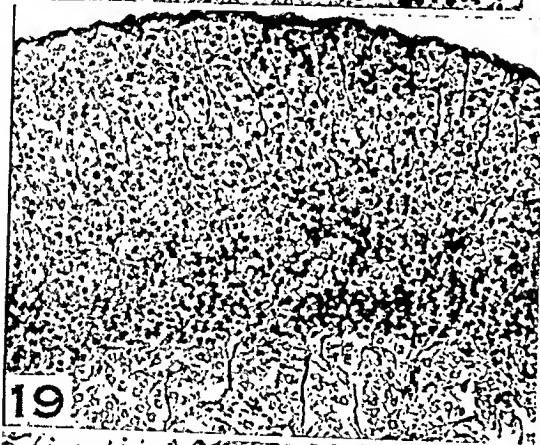
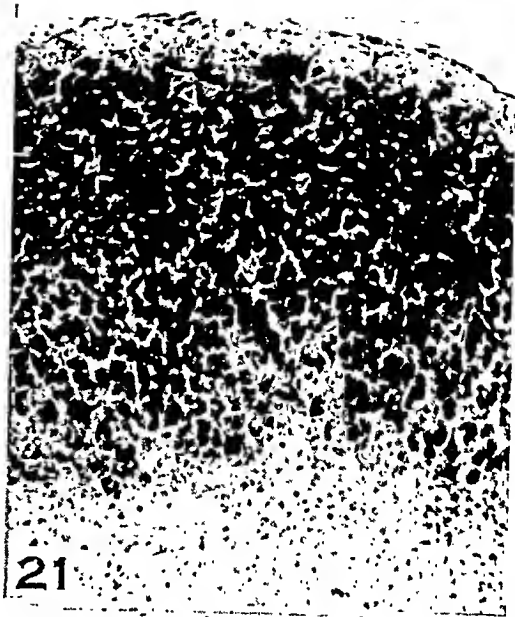
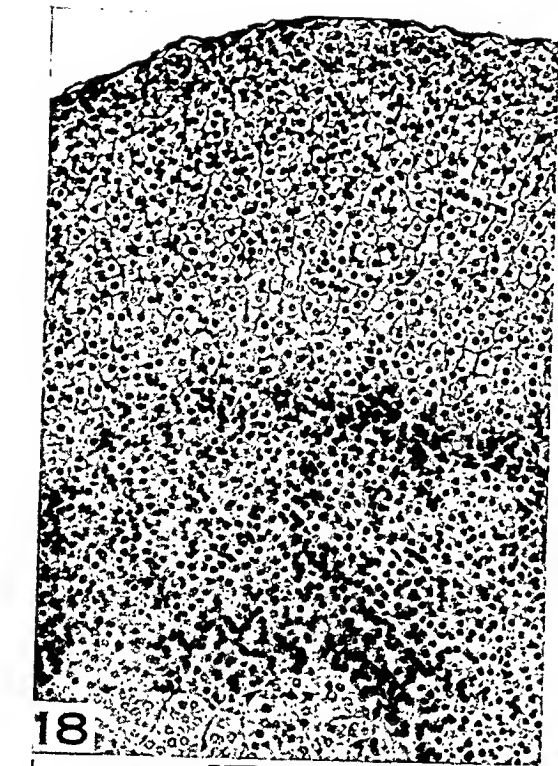


PLATE IV. Adrenals of postpuberally castrated male mice. Figures 18-20 hematoxylin and eosin. Figures 21-23, frozen sections of 15 μ , stained with Sudan IV.

well prevented. The maintenance, however, was not so complete as in those animals injected with Gonadotrophin I. For the most part those cells nearest the medulla were normal, while those more peripherally placed showed signs of degeneration.

The intact female injected with adrenocorticotrophin (ACTH). There is an overall increase in the size of the adrenal cortex which is apparent by inspection, although the adrenals were not weighed. The zona glomerulosa and the zona fasciculata become filled with fine sudanophilic droplets which are Schiff and Schultz positive. The fluorescence is a greenish-blue, spreading over the two zones. The birefringent particles are fine and scattered plentifully in the same region. The increase in size of the cortex is due to the contributions of the zona glomerulosa and the zona fasciculata. The X zone is, in general, narrower than that of the controls. The cells of this zone are in nowise degenerate however. It seems likely that the X zone becomes more compact after ACTH injections, due to the mechanical pressure exerted by the enlargement of the rest of the cortex.

The hypophysectomized female injected with ACTH. In the adrenal of the hypophysectomized female injected with ACTH the zona fasciculata is maintained so that it has all the histological characteristics of that of the normal animal. The zona glomerulosa has not altered its appearance in histological preparations (fig. 4). The histochemical preparations are of interest. Sudanophilic material is maintained in the zona glomerulosa (fig. 7), and this is both Schiff (fig. 13) and Schultz positive. The zone gives a greenish-blue fluorescence and numerous fine birefringent particles are to be found in it (fig. 17). The reactions are strongly positive, more so than in the untreated normal animal but similar to those of the normal treated with ACTH. The

FIG. 18. Untreated castrate. The zona glomerulosa and zona fasciculata are typical. There is no zona reticularis. The secondary X zone which has appeared consequent on castration is wide and deeply staining. A small portion of the medulla is to be seen at the bottom of the photograph.

FIG. 19. Castrate, hypophysectomized for 21 days. The cells of the zona glomerulosa are packed together but have not degenerated. The zona fasciculata is degenerating with vacuolated regions at its inner border and the outer part of the secondary X zone. The secondary X zone has collapsed, the nuclei are pyknotic and there is little cell cytoplasm. The medulla may be seen at the bottom of the photograph.

FIG. 20. Castrate, hypophysectomized for 21 days, Gonadotrophin I for 19 days. The zona glomerulosa is normal, the zona fasciculata is narrow and degenerating. The secondary X zone is wide and well maintained.

FIG. 21. Castrate. The zona glomerulosa is not as strongly sudanophilic as the zona fasciculata. The secondary X zone is not sudanophilic.

FIG. 22. Castrate, hypophysectomized for 21 days. Sudanophilic material has completely disappeared from the zona glomerulosa. The narrow zona fasciculata is densely stained. Lobules of degeneration can be seen in the inner zona fasciculata and in the X zone.

FIG. 23. Castrate, hypophysectomized for 21 days, Gonadotrophin III for 19 days. The zona glomerulosa and zona fasciculata are much the same as in figure 22, above, the hypophysectomized castrate. The zona fasciculata has a narrower and neater appearance perhaps due to the mechanical pressure of the X zone. This latter zone is wide and well maintained.

zona fasciculata reacts similarly to the histochemical tests as that of the normal. The sudanophilic droplets are generally fine, however. In some cases the sudanophilia disappears from the inner region of the zona fasciculata and this condition is illustrated in figure 8. The course of the degeneration of the X zone after hypophysectomy is unaltered by ACTH injections. The lobules of intensely staining sudanophilic material seen in figure 7 arise from the degenerating X zone and, also from cells of the inner part of the zona fasciculata. The degenerating X zone in the ACTH injected animals is more compact than that of the untreated hypophysectomized animal. This may be due to the mechanical pressure of the maintained zona fasciculata.

The spayed female (not hypophysectomized) injected with Gonadotrophin IV (HCG). Gonadotrophin IV (human chorionic gonadotrophin) injected into the spayed female had no detectable effect on the adrenal. Human pregnancy urine fraction or HCG, when injected into the intact virgin mouse, will cause the X zone to degenerate (Takewaki, 1936; Waring, 1942).

The spayed, hypophysectomized female injected with HCG. The adrenals of the mice in this category appeared no different from those of untreated hypophysectomized controls. It would seem that there is no direct action of this gonadotrophin on the adrenal cortex.

The hypophysectomized female injected with HCG. In the adrenals of the mice in this group, the X zone had disappeared and a medullary connective tissue capsule formed² (fig. 5). In the other two zones, the zona glomerulosa and the zona fasciculata, no difference from the course of events after hypophysectomy of the untreated was observed. This indicates that the action of this Gonadotrophin is one on the ovaries without the influence of the animals' own pituitary.

The spayed, hypophysectomized female injected with HCG and with estrogen. The X zone followed the same pattern of degeneration as in the untreated hypophysectomized female, apparently uninfluenced by the preparations injected. Estrogen alone in the same dosage was without effect. The rest of the adrenal was similar to that of the untreated hypophysectomized controls. It would seem that the substance produced from the ovary by HCG which causes X zone destruction is not an estrogen or the synergistic action of the two compounds. The dosage of 1 RU per day may be inadequate. On the other hand, it is doubtful if even large doses of estrogen would cause destruction of the X zone when given over only a short period of, say, 10 days (literature in Waring, 1942; Leathern and Silverman, 1945; Parkes, 1945). Longer periods of treatment would not be comparable

² It may be noted that a primary X zone and a medullary connective tissue capsule do not exist in one and the same adrenal. Normally, in the male, as the X zone disappears with puberty, a medullary connective tissue capsule forms; and, *mutatis mutandis*, in the adrenal of the primiparous female. A medullary connective tissue capsule, however, coexists with the secondary X zone.

to the normal rapid degeneration of the X zone, usually by the 12th day of first pregnancy.

The spayed, hypophysectomized female injected with HCG and progesterone. In these animals the X zone has not disappeared. Progesterone itself in the dosage used does not cause X zone degeneration and does not, even in larger doses, reproduce the effect of pregnancy on the X zone (Martin, 1930; Tolenaar, 1939; Howard and Gengradom, 1940; Waring, 1942; McPhail and Read, 1942; Clausen, 1944). It seems that there is no synergism between HCG and progesterone. Hence, the substance evoked by HCG from the ovaries of the intact or hypophysectomized virgin female is probably neither estrogen or progesterone.

The prepuberally castrated male mouse, normal and hypophysectomized. Castration of the immature male mouse prevents the degeneration of the X zone as the mouse reaches adult age; moreover, the X zone grows in size until it comes to occupy a large percentage of the cortex, as in the virgin female. Such castrates, hypophysectomized at 36 days of age when the X zone has become prominent, show degeneration of the zona fasciculata and X zone by 9 days after operation. The zona glomerulosa presents the same appearance as that of the virgin female described above. This aspect of the prepuberally castrated male mouse has been described and figured in Jones (1949) to which reference should be made. The progress of events after hypophysectomy are no different from those described for the virgin female in this paper with the proviso that the changes are not so far advanced, since the castrates were killed 9 days after operation as against 14 for the virgins.

The hypophysectomized prepuberally castrated male mouse, injected with Gonadotrophin I. This category of mouse injected with the preparation for 7 days shows a maintenance of the X zone, with degeneration of the zona fasciculata and the histochemical changes in the zona glomerulosa in much the same way as in the normal female. No separate illustrations are given, therefore, as they resemble closely figures 3, 9, 12, and 16.

The postpuberally castrated male mouse. Castration of the adult male mouse results in an enlargement of the gland so that 2 months after castration of 2½-month old males, i.e., at 4½ months of age, the pair of adrenals is 51.1 per cent heavier on the average than those of coeval intact males (Table 3). Histological preparations (fig. 18) reveal that an additional layer of cells have appeared surrounding the medulla, and these cells are very similar to those of the X zone, and is termed the "secondary X zone" (Howard, 1939). The adrenal of the normal adult male mouse shows little zona reticularis or none at all (Jones, 1948). It is probable, although not proven, that the new adrenal zone, which arises as a result of castration, is formed by prolifer-

ation of the cells of the inner region of the zona fasciculata where it abuts onto the medullary connective tissue capsule.

The zona fasciculata is strongly sudanophilic, the zona glomerulosa rather less so (fig. 21). The sections prepared after the Schiff reagent, the Schultz method, and for birefringence and fluorescence are very similar to those of the female (figs. 10 and 14), and they are not, therefore, separately figured. The secondary X zone is not sudanophilic, not Schiff positive, nor Schultz positive. It contains no birefringent particles and gives a nonspecific blue-black fluorescence. The adrenal of the postpuberally castrated male mouse some time after castration is, therefore, very similar to that of the young adult virgin female. In the former, however, a medullary connective tissue capsule is present coexisting with the secondary X zone (see footnote 2).

The postpuberally castrated, hypophysectomized male mouse. Hypophysectomy of the castrated male with a well-developed secondary X zone results, by 18 days after operation, in adrenal weight loss of 56 per cent (Table 3). Histological preparations show that the zona glomerulosa is well maintained and that there is considerable degeneration of the zona fasciculata and secondary X zone (fig. 19). After the Sudan stains, the reactions noted as occurring after hypophysectomy in the virgin female, are to be seen (fig. 22), and the pattern after the Schiff reagent, the Schultz method, and with the fluorescence and polarizing microscopes is very similar.

The postpuberally castrated, hypophysectomized mouse injected with Gonadotrophin I or with II. The zona glomerulosa and the zona fasciculata are similar to those zones in the untreated hypophysectomized animal (figs. 20 and 23). The X zone, in contrast to that of the untreated, consists of cells normal in appearance, with round nuclei and eosinophilic cytoplasm (figs. 20 and 23). It is similar in appearance and gives the same reactions as that of the castrate with intact pituitary. This differential maintenance of adrenal zones is reflected in adrenal weights (Table 3). The hypophysectomized animals injected with gonadotrophin have adrenals which are 24.2 per cent heavier than the untreated hypophysectomized animals.

There are some additional points of interest in connection with the postpuberally castrated group of mice. Comparison of the seminal vesicle and ventral prostate weights of the untreated castrates with the normal (Table 3) shows that the former have regressed considerably. There is no evidence from this, therefore, that the adrenal of the castrate animal (and, by inclusion, the secondary X zone) produces a steroid with androgen-like properties. Further, the hypophysectomized animal with the X zone supported by gonadotrophin injections shows no seminal vesicle or prostate stimulation. Another line of evidence comes from the use of the histochemical method of demonstrating alkaline phosphatase. The adrenals of the castrated

male (and of the hypophysectomized and gonadotrophin injected animals) do not show any alkaline phosphatase. The normal male adrenal does so, and the alkaline phosphatase reaction can be brought back to the cortex of the castrate by injections of androgens (Elftmann, 1947). From this it appears that the absence of alkaline phosphatase from the adrenal cortex of the castrated male mouse would indicate that there is no androgen formation.

The estimation of cholesterol (Table 4) shows that in mg per cent, the adrenals of the normal animal and the castrate have about the

TABLE 4. THE MEAN CHOLESTEROL CONTENT OF THE ADRENALS OF NORMAL ADULT AND COEVAL POSTPUBERALLY CASTRATED MALE MICE, CASTRATED FOR TWO MONTHS

	No.	Adrenal wt. mean of pairs	Cholesterol content		
			Total absolute	Total	Free
			mg.	mg. %	mg. %
Normal	5	3.7	.054	1.39	.69
Castrated	5	6.9	.095	1.37	.86

same amount of total and free. In absolute quantities the adrenal of the castrate has about twice as much total cholesterol content as the normal. Since the histochemical preparations indicate that the secondary X zone does not contain cholesterol, it seems likely that, although the permanent cortex of the castrate may have enlarged also, there is in addition a higher cholesterol content in the zona glomerulosa and zona fasciculata as compared to those of the normal.

The one histochemical property which the secondary X zone has in common with the rest of the cortex, is the presence of "ascorbic acid" in the cells. The untreated castrate adrenal, prepared after the method of Deane and Morse (1948), shows fine black granules in the cells of all three zones. The adrenal of the hypophysectomized castrate shows the fine granules in the zona glomerulosa and occasional coarse clumps in the degenerating zona fasciculata and X zone. The adrenal of the hypophysectomized castrate injected with gonadotrophin shows fine black granules in the cells of the zona glomerulosa and the X zone; the zona fasciculata has scattered coarse black clumps. The interpretation of this finding is not clear. It does bring out the point that the gonadotrophin seems to maintain the X zone in a normal condition. The reducing substances revealed may be ascorbic acid, but the connection of this substance with adrenal cortical physiology is conjectural (Long, 1946).

DISCUSSION

The traditional zoning of the mammalian adrenal cortex on histological grounds may well be paralleled by divisions of physiological

function. In the mouse at least, it is clear that three zones, the zona glomerulosa, the zona fasciculata and the X zone have different relationships with the pituitary gland. The zona glomerulosa does not degenerate after removal of the pituitary. Indeed, it continues to divide so that in the long-term hypophysectomized animal the zone is quite wide (Jones, 1950). Although this property is independent of the hypophysis, nevertheless, the zone is not completely free of pituitary control. In the ACTH injected, hypophysectomized animal, acetone-soluble material (which is sudanophilic, Schiff and Schultz positive, possesses a specific fluorescence and is birefringent) is plentifully scattered in the zona glomerulosa. In the untreated animal this material disappears from the zone. It is apparent from the discussion of the tests used (Dempsey, 1948; Jones, 1949, 1950; Yoffey and Baxter, 1949) that they cannot be taken as rigorously specific for one class of compound. They reveal substances, nevertheless, related to the hormonal activity of the gland. It would follow, therefore, that adrenocorticotrophin plays a role, as yet undetermined, in the regulation of the zona glomerulosa.

While the complete dependence of the zona fasciculata on ACTH requires no comment, the X zone adds yet another variant to the pituitary-adrenal relationship. It is apparent that the factor trophic to the latter zone is not ACTH but a gonadotrophin. The gonadotrophic preparations used were not pure so that it is impossible to say whether luteinizing hormone (LH), or follicle stimulating hormone (FSH), or a synergistic reaction between the two, is responsible for the effect on the X zone. It would seem that the balance of evidence is in favor of considering the X zone-trophic factor as LH. Certainly the degree of maintenance of the X zone was directly related to the LH potency of the preparation employed. Further, Greep and Jones (1950) showed that rat pituitaries, LH in nature, maintained the X zone when injected into immature hypophysectomized female mice while rat pituitaries, FSH in type did not.

If the X zone maintaining factor is LH then it seems that in the male the androgens produced by the gonadotrophin from the interstitial cells act directly on the zone (Jones, 1949). Their destructive action over-rides the maintaining effect of the LH. If, however, the animal is castrated before puberty then the pituitary LH continues to act on the X zone, without the intervention of androgens, causing its growth. In the virgin female the increase in size of the X zone up to puberty and its persistence beyond is not surprising, if the zone is dependent on LH. The reason for its gradual degeneration later in adult life is not clear. It is possible that there is a lessening of LH secretion with age, although a sufficient level is maintained for the continuance of the estrous cycle. It is doubtful if androgens of ovarian origin are responsible because the course of degeneration is unaltered by ovariectomy (Howard, 1927; Deanesly, 1928). The possibility

exists that the adrenal steroid hormones may have a very slight androgenic effect sufficient to cause gradual degeneration of the zone.

The mechanism of the rapid degeneration of the X zone during first pregnancy is not known. Efforts to attribute this to estrogen or to progesterone or to their concerted action have failed (Howard and Grengradom, 1940; Waring, 1942). Furthermore, the X zone of the pseudopregnant animal—achieved by sterile mating (Takewaki, 1939) or by luteotrophin (Jones, unpublished)—does not degenerate. Nor, apparently, does the formation of deciduoma affect the zone (Takewaki). Removal of the embryos however, at the relevant time prevents or arrests the X zone degeneration (Takewaki, 1936). The experimental use of HCG in this respect may not be relevant to the mouse as the rodent placenta does not produce this type of gonadotrophin (Astwood and Greep, 1938). The difference in action between HCG, which has some luteinizing hormone properties, and LH, is marked. The former produces a substance from the mouse ovary, without pituitary mediation, which acts directly on the X zone to cause it to degenerate. This substance is probably neither estrogen or progesterone. Takewaki (1935) suggested that a third ovarian hormone accounts for this and Starkey and Schmidt (1938) that an ovarian androgen is responsible. In the consideration of the degeneration of the zone during first pregnancy some confusion has arisen because it is generally believed that implantation is *not* necessary for this to occur, and therefore no analogy may be drawn from the action of HCG. However, reexamination of the literature (vide Tamura, 1926; Howard, 1927; Deanesly, 1928; Takewaki, 1936; Parkes, 1945) reveals that the general rule is for X zone degeneration to take place between days 7 and 12 of first pregnancy. Implantation in the mouse takes place on day 5 or 6 (Snell, 1946). It may well be that variation in the time of onset of X zone degeneration would, if the facts were available, be found to vary in the same way as time of implantation. Hence the difference between the pseudopregnant and the pregnant mouse in terms of hormones, would lie in an additional foetal-endometrial hormone present in the latter. This acting directly or via the ovary would cause X zone degeneration. More research on this subject may well reveal widely applicable findings on the hormones of pregnancy.

It appears that castration of the adult mouse allows a gonadotrophin to act upon adrenal cortical cells to produce a secondary X zone. In the normal mouse no response may be elicited in the presence of circulating androgens. From the gonadotrophin preparations used there is some indication that LH may be the factor involved, although, as in the case of the primary X zone, FSH or a synergism of the two cannot be excluded. Certainly castration of the rat results in increase in stored LH (Hellbaum and Greep, 1940). Parabiosis experiments with joined castrated male-hypophysectomized male indicate that

some of the LH is secreted as evidenced by the androgen stimulation of the prostate and the seminal vesicles of the hypophysectomized partner (Cutuly, McCullagh and Cutuly, 1937). The chief factors in the evocation of the secondary X zone seem to be a steady secretion of LH and the absence of circulating androgens. The castrate male would appear to fulfill these conditions.

How far these findings in the mouse may be applied to the mammalia as a whole is not known. In the human, the fetal cortex has been thought to be similar to the mouse X zone (Grollman, 1936). The fetal cortex begins to degenerate soon after birth and normally seems to have no physiological role. Under abnormal conditions, in the adreno-genital syndrome, the production of androgens has been attributed to it (see Parkes, 1945). It is probable that both the mouse X zone and the fetal cortex develop embryologically without hormonal influence.³ In the mouse the X zone which differentiates out embryologically (Waring, 1935) becomes competent to respond to LH. Normally the human fetal cortex degenerates before this is possible. It could be envisaged in the human, that under abnormal conditions the pituitary may produce LH at a much earlier age when the unde-generated fetal cortex was able to respond. This response could be, perhaps, the production of androgens to account for at least one kind of prepuberal adreno-genital syndrome. In the mouse the X zone normally comes under the influence of LH but there is no evidence of androgen production from this zone (Gersh and Grollman, 1939a and 1939b; Howard, 1946). The X zone may be regarded as a potential androgen producing layer which under certain abnormal physiological conditions might be able to turn over to steroid hormone production. The conditions for this are not known but it is interesting to note that the long-term castrated male mouse produces sexogens from adrenal neoplasms (Wooley and Little, 1945a & b).

SUMMARY

Three zones of the mouse adrenal cortex, the zona glomerulosa, the zona fasciculata and the X zone each have a different relationship to the secretions of the anterior pituitary. The zona glomerulosa is capable of an existence independent of the hypophysis but the presence of acetone-soluble sudanophilic material related to hormone activity within the zone depends on adrenocorticotrophin. The zona fasciculata is completely dependent on adrenocorticotrophin. The X zone is dependent on a gonadotrophin, possibly LH in nature. Similarly, the secondary X zone which arises in the postpuberally castrated male mouse, is dependent on a pituitary gonadotrophin, possibly LH in nature.

³ The absence of the X zone from the dwarf mice of Deanesly (1938) may be due to embryological reasons and not to some postnatal hormone deficiency.

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PERIODIC UTERINE BLEEDING IN OVARI-ECTOMIZED MONKEYS GIVEN CONSTANT DAILY DOSES OF OESTROGEN

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UTERINE bleeding does not take place during the administration of adequate doses of oestrogen to ovariectomized rhesus monkeys, but only as a withdrawal effect some days after treatment has been stopped. If the dose is inadequate bleeding occurs neither during nor after the end of oestrogen treatment. On the other hand, it takes place periodically during a course of treatment in which the animals are maintained on a constant intermediate level of the hormone (Zuckerman, 1937, 1940), the intervals between successive phases of bleeding being referred to for convenience as "artificial threshold cycles" or as "threshold cycles." This observation bears significantly on explanations of the hormonal control of normal menstrual bleeding.

The present paper shows that a succession of such cycles may be induced over a period of several years, and that they are as readily induced by stilboestrol as by oestrone.

MATERIAL AND METHODS

Ten ovariectomised rhesus monkeys (*Macaca mulatta*) were used in this study. Details about the animals and the experiments are given in Table 1. Daily injections of oestrogen were given intramuscularly in arachis oil solution. Daily vaginal lavages were made to detect the occurrence of uterine bleeding.

In establishing the level of stimulation necessary to cause recurrent phases of bleeding, an arbitrary amount of oestrogen was injected daily, usually for not less than 100 days. If bleeding did not occur during this period, the injections were stopped. If withdrawal bleeding then occurred, the injections were re-started at a lower level. If it did not occur, the daily dose was raised. In either case, the procedure was continued until bleeding occurred during the course of injections.

RESULTS

Oestrone

Threshold cycles were successfully induced in three of seven monkeys treated with oestrone. They occurred occasionally in three others, and not at all in the seventh.

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The experiment was most successful in two young mature monkeys (OM 536, 537) that had not been experimented on before, and in whom treatment was begun within a few weeks of the removal of their ovaries. OM 536 underwent 27 successive cycles in 1088 days on 10 μ g. oestrone daily (mean cycle length 40.3 ± 3.93 days) before failing to respond. Four more cycles occurred on daily doses of 20

TABLE 1. DETAILS OF ANIMALS USED

Animal No.	Total amount of oestrogen (mg.)	Days under experiment	Body-wt. at start (g.)	Body-wt. at end (g.)	Previous history
71	7.534	1198	6100	7540	Mature. 43 phases of experimental oestrogenic stimulation.
452	4.891	866	4060	5760	Young mature. No previous treatment.
461	4.388	829	4120	6580	Young mature. No previous treatment.
382	15.640	1600	6320	7400	Mature. 24 phases of experimental oestrogenic stimulation.
69	7.282	697	7750	7280	Mature. 43 phases of experimental oestrogenic stimulation.
533	9.786	916	4160	4140*	Adolescent. 5 phases of experimental oestrogenic stimulation.
536	15.940	1469	3800	7000	Prepubertal. No previous treatment.
537	12.10	1287	3720	8820	Prepubertal. No previous treatment.
447	13.983	1879	2600	5600	Prepubertal. No previous treatment.
430	20.499	1782	4020	8000	Adolescent. 8 phases of experimental oestrogenic stimulation

* The maximum body-weight reached during the experiment was 6780 g. Subsequently, however, the animal lost weight.

μ g. and two more on 15 μ g. The experiment was ended on the 1469th day.

OM 537 underwent 26 successive cycles in 1112 days on 10 μ g. oestrone daily (mean cycle length 42.8 ± 6.10 days) before a period of 89 days' amenorrhoea occurred and the experiment was discontinued.

In the third successful experiment (OM 382) twelve cycles occurred in a period of 303 days on 10 μ g. oestrone daily. These were followed by a phase of amenorrhoea which lasted 86 days. The dose of oestrone was then lowered to 8 μ g. but no bleeding occurred during the 110 days it was maintained at this level. Bleeding occurred again when the dose was raised to 10 μ g. daily (3 cycles in 236 days). The dose was then lowered to 9 μ g. daily, for a period of 184 days, in which 6 cycles occurred. In the following 319 days it was kept at 10 μ g. and 9 cycles occurred. Withdrawal bleeding followed the cessation of injections (on the 1600th day of the experiment) and the monkey was autopsied 30 days later. The uterus was small, and on micro-

scopic examination the endometrium appeared thin. It contained numerous straight glands embedded in a dense stroma.

The earlier history of two of the three monkeys in which the experiment was partially successful has already been recorded (OM 477 and OM 430, Zuckerman, 1940) OM 447 began with 6 threshold cycles on 10 μ g. oestrone daily. A period of 553 days' amenorrhoea followed, which was interrupted by one day's bleeding on the 200th day. During this long phase of amenorrhoea the level of oestrogen treatment was gradually dropped to 7 μ g. for 82 days, 5 μ g. for 223 days, and 3 μ g. for 131 days. Uterine bleeding did not occur when the injections were stopped.

It was thought that the uterus might have become insensitive during this long period of treatment with sub-threshold quantities of oestrogen. The animal was therefore given 100 μ g. oestrone daily for 10 days. Withdrawal bleeding started 7 days later. The animal was then put on to 10 μ g. oestrone daily. Three threshold cycles occurred, before amenorrhoea once again supervened. Further attempts to establish recurrent threshold cycles on 8 μ g., 8.5 μ g., 10 μ g. and 11 μ g. oestrone daily were unsuccessful, and the experiment was ended on the 1879th day.

The first four threshold cycles by OM 430 already recorded occurred at a level of 10 μ g. oestrone daily. Amenorrhoea then supervened, but cycles were re-established temporarily on 8 μ g. daily. A second period of amenorrhoea lasting 157 days ensued, and subsequent daily doses of 5 μ g. (for 63 days) and of 10 μ g. (for 65 days) did not induce bleeding. Cycles were re-established at a level of 15 μ g., seven occurring during a period of 527 days. 112 days' amenorrhoea followed, presumably due to an excessive amount of oestrogen, since withdrawal bleeding followed the cessation of the daily injections. The experiment was resumed at a daily dose of 13 μ g., but bleeding did not occur during the 113 days of the experiment, nor as a withdrawal effect when the injections were again stopped. They were then resumed but threshold cycles could not be re-established either on 14 μ g., 12 μ g. or again on 15 μ g. daily, and the experiment was ended on the 1782nd day.

Only a few threshold cycles were induced in the third monkey (OM 533) in the group of partially successful experiments. One of 65 and one of 96 days occurred during each of two courses of injections of 10 μ g. oestrone daily. Both ended with periods of amenorrhoea lasting over 100 days, and neither was followed by withdrawal bleeding. Two threshold cycles of 26 and 31 days were then obtained on 12 μ g. daily, and three (22, 26 and 87 days) on 15 μ g. before amenorrhoea again set in. Withdrawal bleeding occurred 9 days after the 15 μ g. injections were stopped. One further cycle was obtained on 13 μ g. daily. The animal was autopsied on the 17th day of the subsequent incomplete cycle. The uterus was normal in size. The endo-

metrium appeared thin and inactive and contained a number of straight glands, in a non-oedematous stroma.

Treatment was continued for a total of 697 days in the one completely unsuccessful experiment (OM 69). Bleeding did not occur at dose levels of 8 μ g. (for 119 days), 10 μ g. (for 133 days), 15 μ g. (for 68 days), and 20 μ g. (for 150 days). Bleeding occurred seven days after the 20 μ g. injections were stopped. The monkey was autopsied 35 days after the last injection.

The uterus was normal in size. Parts of the endometrial stroma were replaced with fibrous tissue, but in general it was moderately oedematous and contained straight glands in which there were several mitotic figures. The vaginal epithelium was deeply stratified.

Stilboestrol

Three monkeys (OM 71, OM 452, OM 461) were used in attempts to establish threshold cycles of uterine bleeding on constant daily doses of stilboestrol.

In the first (OM 71) six cycles were obtained on 10 μ g. daily. Withdrawal bleeding followed the cessation of these injections, and of subsequent courses of injections of 7.5, 5.0, 3.5, 2.5 and 2.0 μ g. daily. Two cycles in a period of 89 days were obtained at a level of 1.5 μ g. stilboestrol daily. The monkey was autopsied 31 days after the last injection. The uterus was normal in size. The endometrium was thin and there was an exceptionally dense condensation of stroma under the epithelial lining of the cavum uteri. There were only a few straight glands in which some mitoses could be seen.

The amounts of stilboestrol given to OM 452 were progressively reduced from 10 μ g. to 3.5 μ g. daily. At this level 10 successive cycles were obtained in a period of 421 days. In the third and last animal (OM 461) a single cycle occurred at an injection level of 10 μ g. daily. Cycles did not recur until the dose was reduced to 2.5 μ g. daily. At this level 10 successive cycles were obtained in a period of 240 days.

ANALYSIS OF THE RESULTS

Tables 2 and 3 show the mean length of the threshold cycles and the duration of bleeding for the dosages of hormone employed.

The dose of oestrogen necessary to produce threshold cycles is variable and not easy to determine. As previously reported (Zuckerman, 1940), between 5 and 20 μ g. are required daily in the case of oestrone. Stilboestrol is three to four times more active. Cycles seem to be established more easily in younger animals with little or no previous experimental oestrogen treatment than in older monkeys whose uteri may have undergone abnormal changes.

There is clearly no systematic relation between the amount of hormone given and the mean cycle length. The wide variation in cycle-length seems to depend on differences in responsiveness be-

TABLE 2. CYCLES OBTAINED WITH THRESHOLD DOSES OF OESTRONS DAILY

Dose of hormone μ g. daily	No. of monkeys used	No. of cycles obtained	Mean length of cycles (days) \pm s.e.m.	Mean duration of bleeding (days) \pm s.e.m.
20	1	5	31.2 ± 5.0	7.2 ± 0.8
15	4	13	57.4 ± 8.2	8.7 ± 0.7
13	1	1	$26.0 \pm \text{—}$	$7.0 \pm \text{—}$
12	1	2	28.0 ± 3.0	$6.0 \pm \text{—}$
11	1	1	$27.0 \pm \text{—}$	$2.0 \pm \text{—}$
10	5	79	42.1 ± 2.9	6.1 ± 0.3
9	1	6	30.7 ± 5.3	5.2 ± 0.7
8	2	7	45.0 ± 7.4	4.6 ± 1.1
5	1	1	$23.0 \pm \text{—}$	$4.0 \pm \text{—}$
All experiments: 115			42.3 ± 2.3	6.2 ± 0.25

tween animals and within an individual animal from time to time. For example, threshold cycles were established at three different times in one monkey (OM 382) at a level of 10 μ g. oestrone. In the first experiment the mean length of 12 cycles was 23.2 days, and of the duration of bleeding 5.1 days. The corresponding figures for the second series (4 cycles) were 78.7 and 1.7 days, and for the third (9 cycles) 35.4 and 5.2 days.

TABLE 3. CYCLES OBTAINED WITH THRESHOLD DOSES OF STILBOESTROL DAILY

Dose of hormone μ g. daily	No. of monkeys used	No. of cycles obtained	Mean length of cycles (days) \pm s.e.m.	Mean duration of bleeding (days) \pm s.e.m.
10	2	7	56.3 ± 9.2	8.0 ± 1.6
3.5	1	10	42.1 ± 4.5	11.4 ± 2.1
2.5	1	10	24.0 ± 2.2	8.4 ± 1.0
1.5	1	2	44.5 ± 10.5	2.5 ± 0.5
All experiments: 29			39.5 ± 3.7	8.9 ± 0.97

The mean length of all the 115 threshold cycles induced by oestrone (42.3 ± 2.3 days) or of the 79 cycles which occurred at the level of 10 μ g. oestrone (42.1 ± 2.9 days) is very close to the average figure of 43 ± 2.6 days previously reported for a smaller series of observations (Zuckerman, 1940). The duration of bleeding is, however, shorter (6.2 ± 0.25 days) as compared with 7.0 ± 0.1 days (Zuckerman, 1940). Despite great variation from one monkey to another, the mean length of the stilboestrol cycles (39.5 ± 3.7 days) is also close to the figures already recorded for cycles induced by oestrone, though the duration of bleeding is somewhat longer (8.9 ± 0.97 days). Besides being much more variable, the length of the experimental cycles is considerably greater than in normal menstrual cycles.

DISCUSSION

The present findings confirm the observation that periodic fluctuations in the responsiveness of the uterus occur in the course of constant

daily treatment with oestrogen. Threshold artificial cycles were, for example, obtained without interruption in two monkeys over a period as long as 3 years. It seems reasonable to suppose, therefore, that periodic changes in uterine sensitivity also play a part in the control of the normal menstrual rhythm.

Corresponding fluctuations in sensitivity have been observed in the vagina. They occur in rats treated with a constant daily dose of oestrogen (del Castillo & Calatroni, 1930; Wade & Doisy, 1935; Bourne & Zuckerman, 1941; del Castillo & Di Paola, 1942), and take place in phase with cyclical changes in the size of the adrenal cortical cells (Bourne & Zuckerman, 1941). The fact that the adrenal changes also occur in hypophysectomized animals suggests that the rhythmic changes in the cortex may be primary to changes in the sensitivity of the reproductive tract to oestrogen, or a cause of cyclical variation in the total amount of circulating steroid hormone. Preliminary experiments to test this hypothesis in monkeys (Zuckerman, 1940) were unsuccessful owing to operative mortality.

Gilbert & Gillman (1944) have also reported that cyclical fluctuations occur in the size of the sexual skin of baboons maintained at a constant level of oestrogenic stimulation. Despite the fact that cyclical changes do occur in the adrenal cortex, in at least the rat, in phase with those that occur in the reproductive organs, they have attempted to explain their observation without reference to a possible adrenal mechanism. They suggest that the active sex skin requires more oestrogen to maintain it in a swollen condition than is needed to induce swelling at the start. When this extra amount is lacking, as would occur on a constant level of stimulation, the swelling declines until a store of oestrogen is built up sufficient to restimulate swelling.

The occurrence of uterine bleeding in ovariectomized women during a course of oestrogen injections has sometimes been taken to throw doubt on the accepted view that menstrual bleeding is generally due to an oestrogen-withdrawal effect. Occasional observations reported by Bishop (1938), Reynolds, Kaminester & Schloss (1940), and Di Paola & del Castillo (1942), however, show that threshold artificial cycles of the kind discussed in this paper also occur in women, and resemble those obtained in monkeys in that they are rather irregular and longer than normal.

SUMMARY

Periodic uterine bleeding occurs in spayed monkeys injected daily with constant threshold amounts of oestrone. In two monkeys such threshold cycles have occurred without interruption for as long as three years.

Stilboestrol produces the same effect in doses one-third to one-quarter that of oestrone.

The experimental inter-menstrual period (40-42 days) is longer and more variable than the normal menstrual cycle.

ACKNOWLEDGMENTS

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NOTES AND COMMENTS

ACCELERATED GROWTH WITH SEXUAL PRECOCITY IN FEMALE MONKEYS RECEIVING TESTOSTERONE PROPIONATE¹

IT HAS been possible to double the growth rate and reduce by half the age of the young female monkey at time of menarche (first menstruation). The androgen, testosterone propionate,² was the agent used and the animal, *Macaca mulatta*, or rhesus monkey.

In a rather well-standardized monkey colony of five generations, the age at which the monkeys mature is about two years, when they weigh around 3500 grams. Crystalline testosterone propionate dissolved in vegetable oil was given intramuscularly to two young monkeys weighing 1410 and 1600 grams. The dosage was 7.5 mg. per kilo per week.³ Hormone treatment began at five months, inducing an immediate rise from the normal curves for body weight and length (see graphs). At one year the treated animals reached the weight and length of two-year-olds and menarche occurred exactly at the end of a year in one animal, and at one year nineteen days in the other. Androgen treatment was then discontinued.

This was a true beginning of sexual maturity and not just a spurious uterine bleeding, for six and seven menstrual cycles, somewhat irregular as typical of adolescence, have followed, accompanied by the external skin changes characteristic of the maturing monkey. This great augmentation of growth and differentiation of tissues has not been analyzed. At present one is inclined to interpret it as a general anabolic effect of the nitrogen retention action of the androgen but the possible release of growth hormone, of increase in hypophyseal hormones, protein in nature, or some other intervening mechanism may contribute. The future of the individual animal and tissues which have been subjected to this great acceleration in growth rate is of interest and possible importance.

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² Oreton, supplied by Schering Corporation.

³ Earlier work with the male monkey indicated that this dosage might be optimal. van Wageningen, Fed. Proc. 6: 219. 1947

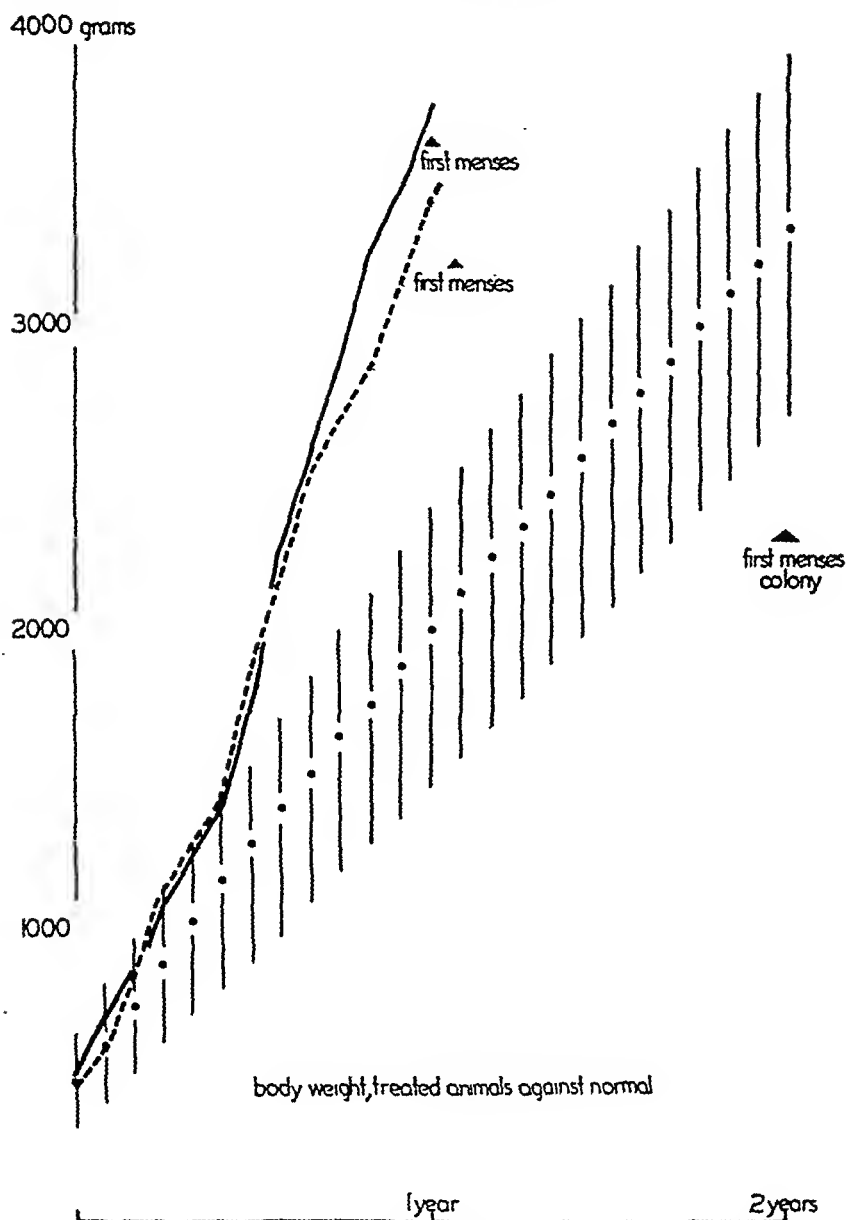


FIG. 1. Individual weight curves of the two hormone-treated animals shown with the span of curves of twenty normal young monkeys recently born and raised in the colony.

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A STUDY OF THE ORIGIN AND DISTRIBUTION OF THE ANTIGONADOTROPHIC SUBSTANCE IN ANIMALS CHRONICALLY TREATED WITH CRUDE SHEEP PITUITARY EXTRACT¹

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SOME few attempts have been made to determine the site of formation and the location of antihormones outside of the blood stream where they are readily demonstrable. Zondek and Sulman (1942) reported that urine and extract of liver, spleen, muscle, ovary, and testis of animals which had received repeated injections of prolan were lacking in antigonadotrophic activity. On the other hand de Fremery and Scheygrond (1941) were able to demonstrate antiprolan in the milk of a pregnant goat which had been protractedly treated with prolan. Gordon, Kleinberg, and Charipper (1939) finding that blockage of the reticuloendothelial system or splenectomy interfered with antihormone formation concluded that the reticuloendothelial system participated in the production of antigonadotrophic substances as would appear to be the case for other antibodies.

It is now generally agreed that antihormones are antibody-like in nature (see Thompson, 1941), hence it may be assumed that the problem of the origin and location of antihormones in the animal body becomes one with that of antibodies in general. Within recent years an extensive literature has accumulated which points to the lymphoid tissue as a whole and the lymphocyte in particular as the source of antibody, and evidence has been given to show that the release of antibody from lymphoid tissues is regulated by the pituitary gland through its effect upon the secretions of the adrenal cortex.

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Inclusive summaries dealing with the question have been presented by Ehrich and Harris (1945), White and Dougherty (1946), and Dougherty and White (1947).

The object of the work to be reported here has been to establish whether lymphoid tissue or more specifically the lymphocyte is the source and site of formation of the antigonadotrophic substance. This problem has been approached by the direct method of extraction of tissues and experimental modification of lymphoid tissues of immunized animals.

MATERIALS AND METHODS

Sexually mature young adult rabbits of various breeds and male rats of 60-90 days of age of the Sprague-Dawley strain were used for the production of the antigonadotrophic substance. These animals were given daily subcutaneous injections of 250-500 mg.³ of crude sheep pituitary extract prepared according to the method of McShan and Meyer (1943). At intervals after the initial injection blood samples were taken from the heart of the treated animals. The serum was recovered and assayed for the presence of antigonadotrophic activity. The assay methods employed here were similar in principle to those which are usually used in studies of antigonadotrophic serums. Essentially the methods involve the inhibition of gonadotrophic extract by antigonadotrophic substances injected simultaneously but at separate site into 21 day old female rats. By one method the ovarian weights are used as the criterion of the effect in which case a total dose of 100 mg. of pituitary extract is given; by the other method the weights of the uteri are used as the index of activity, and in this case a total dose of 5 mg. of the extract is injected. With the exception of the assay of antigonadotrophic activity of the lymph plasma presented in Table 1 all assays were by use of the uterine weight method.

Animals which showed high antiserum titers were considered suitable for study. Lymph plasma, lymphocytes, lymph nodes, spleen, thymus, bone marrow, liver and cecum from them were assayed for possible antigonadotrophic effects.

Lymphocytes and lymph plasma were collected by cannulation of the thoracic duct of immunized rabbits. The lymph was collected in graduated centrifuge tubes as it came from the cannula and heparin was added at intervals to prevent clotting. It was centrifuged at constant speed for a definite time after which the lymphocyte volumes were read. Because of the frequent appearance of small amounts of clotted material in the lymph these measurements could be considered only rough approximations of the actual volume. However, this was considered satisfactory for the qualitative study intended.

During some of the early operations smears were made of cells collected by this method and red cell counts carried out on the lymph as it came from the cannula. The smears showed that the lymphocytes were overwhelmingly predominant. Red blood cells although invariably present in small numbers were not in sufficient concentration to indicate contamination from the blood stream.

³ Indicates in all cases milligram equivalents of original pituitary powder used in making the preparation.

In some cases the lymphocytes were washed 3 times in saline and in others as much of the fluid as possible was removed from the tube by blotting after the supernatant fluid had been decanted.

Both lymph plasma and lymphocytes were stored in the frozen state until assayed. For this purpose sufficient normal saline was added to the lymphocytes to provide for 6 injections of .5 ml. for each assay animal.

Tissues to be assayed for antiserum content were removed at autopsy and frequently kept frozen until used. They were prepared for injection by homogenizing in 2 times their weight of normal saline. Where possible the entire homogenate was injected, but where this proved impossible due to clotting of the homogenized tissue only the supernatant fluid was injected.

In addition to the direct assay methods outlined above attempts were made to ascertain the location of the antigonadotrophic substance indirectly by the use of adrenal cortical materials. Antihormone animals were injected with various amounts of Adrenal Cortex Extract (supplied by the Upjohn Co.) and at intervals after injection the serum was tested for changes in the antigonadotrophic titer. These serums were assayed in total amounts ranging from .3 ml. to .005 ml.

A second indirect approach was applied. Since the enhancement of antibody titer after adrenal cortical treatment was attributed by White and Dougherty (1946) to the breakdown of lymphocytes and consequent release of immune substances, any agent which causes the destruction of lymphocytes should bring about increased antibody in the serum. A lymph node antiserum was considered to be such a substance.

To prepare this serum a large rabbit was injected 9 times intravenously with fresh rat lymph node homogenized in 2 times its weight of normal saline. The first 2 injections were of .1 ml. each, the following 6 of .4 ml. and the last of .5 ml. The rabbit was bled on the fifth day after the last injection. This antiserum was administered intracardially to antihormone rats under light ether anesthesia after having been mixed with an equal part of heparin.

Total white cell counts and differential counts were done before and after this treatment, and samples of blood were withdrawn from the heart for antigonadotrophic assay.

Finally, the popliteal lymph node was employed in a manner suggested by the work of Ehrlich and Harris (1942). For this study various amounts of pituitary extract were injected into the sole of the foot of rats and rabbits and at intervals the popliteal lymph nodes of rabbits or popliteal and iliac lymph nodes of rats were removed and assayed by the method outlined above for other tissues. The particular advantage of such a technique is considered to lie in the fact that the injected material is for the most part required to pass through these particular nodes. As a result antibodies might be expected to develop more rapidly and be more concentrated in them.

Two series of rabbits were given a single injection of pituitary extract. On the 4th, 6th, and 8th day the animals were killed and the popliteal lymph nodes were removed and assayed for antigonadotrophic activity. In another series one animal received 2 injections of extract 6 days apart, autopsy being performed on the 10th day after the first injection and the 4th day after the last injection.

Similar experiments were carried out using rats with the difference that both popliteal and iliac lymph nodes from 3 animals were pooled for assay on the 4th, 6th, and 9th day after the last injection.

RESULTS AND DISCUSSION

Lymphocytes and Lymph Plasma

Table 1 gives the results of assays of lymphocytes collected by cannulation of the thoracic duct in 12 antihormone and 3 normal rabbits. The data obtained from assays of unwashed cells from 3 animals has been averaged for presentation in tabular form, and other groups have been arranged on the basis of the volumes of lymphocytes assayed. The uterine weights of immature females receiving

TABLE 1. ASSAY OF LYMPHOCYTES AND LYMPH PLASMA OF NORMAL AND ANTIHORMONE RABBITS FOR ANTIGONADOTROPHIC ACTIVITY

No. animals cannu- lated	Lymph plasma (ov. wgt.) mg.	Control (ov. wgt.) mg.	Lymphocyte volume ml.	Lymphocytes (ut. wgt.) mg.	Control (ut. wgt.) mg.
Antihormone animals					
3	24 (3)*	60 to 80	.7-.25	34 (3)†	40 (4)
2	19 (2)		.35-.3	38 (2)	39 (6)
1	19 (1)		.2	34 (1)	40 (4)
2	—		.2	68 (2)	50 (3)
3	27 (3)		.15-.12	52 (3)	38 (9)
1	29 (1)		.05	39 (1)	52 (3)
Normal animals					
3	—	—	.35-.3	48 (4)	39 (6)

* Numbers in parentheses indicate number of assay animals.

† Indicates unwashed cells.

both lymphocytes and 5 mg. of pituitary extract may be compared with uterine weights of animals receiving only 5 mg. of pituitary extract. The antigonadotrophic titer of the lymph plasma from which these cells were separated is given to show that all animals had reasonably good antigonadotrophic titers at the time the lymph was collected. A total dose of 2 ml. of lymph was used to assay lymph plasma by the ovarian weight method.

It will be seen that there is no indication of antigonadotrophic activity in the lymphocytes.

TABLE 2. COMPARISON OF ANTIGONADOTROPHIC ACTIVITY OF THE BLOOD SERUM AND LYMPH PLASMA FROM THE SAME RABBIT

Dose (ml.)	Blood		Lymph	
	(Ut. wgt. mg.)	(Range mg.)	(Ut. wgt. mg.)	(Range mg.)
.15	26 (9)*	18-42	28 (7)	18-36
.08	37 (7)	26-43	31 (9)	21-41
.04	29 (7)	20-41	32 (7)	20-45
.02	40 (7)	23-77	34 (7)	23-45
.01	54 (5)	34-75	47 (5)	34-65
.005	67 (5)	61-73	54 (5)	44-76
	Control = 41 (19)		Control = 37 (13)	

* Numbers in parentheses indicate number of assay animals.

Further assays of lymph plasma using the uterine weight method were made for the purpose of comparing it with blood serum with regard to antigonadotrophic properties. The results are presented in Table 2. Here the dose refers to the amount of antigonadotrophic lymph plasma and blood serum given with the usual 5 mg. of pituitary extract. The effect of the 2 fluids at different dosage levels is quite similar. And, as has been frequently used by us (unpublished data), both fluids become augmentative at the lower doses resulting in uterine weights greater than those for controls.

Tissues

Data showing the absence of the antigonadotrophic substance in homogenates of tissues from antihormone and normal rats and rabbits are given in Table 3. The dose is in terms of the milligrams of tissue homogenized in saline and injected simultaneously but at separate site from the gonadotrophin. Tissues from both antihormone and

TABLE 3. NORMAL AND ANTIHORMONE RABBIT AND RAT TISSUES ASSAYED FOR ANTIGONADOTROPHIC ACTIVITY

Tissue	Dose* (mg.)	Antihormone		Normal	
		Exptl. (Ut. wgt. mg.)	Control (mg.)	Exptl. (Ut. wgt. mg.)	Control (mg.)
Rabbit					
Lymph Node	600-350	57 (4)†	35 (4)	45 (8)	37 (5)
Lymph Node	300-250	78 (6)	50 (3)	—	—
Spleen	450-250	52 (9)	41 (7)	60 (5)	44 (8)
Liver	800-300	58 (6)	37 (6)	50 (2)	39 (2)
Bone Marrow	500	79 (3)	50 (3)	70 (3)	50 (3)
Cecum	500	45 (4)	37 (2)	—	—
Thymus	400	—	—	51 (2)	39 (2)
Rat					
Lymph Node	400-350	53 (7)	40 (7)	56 (4)	49 (5)
Lymph Node	200-100	44 (6)	34 (5)	—	—
Spleen	1000-800	41 (6)	34 (4)	—	—
Liver	700-400	51 (7)	38 (7)	55 (5)	43 (5)
Thymus	350-100	52 (4)	35 (4)	54 (2)	39 (2)

* Quantity of tissue injected.

† Numbers in parentheses indicate number of assay animals.

normal animals are compared with regard to their effect on the activity of 5 mg. of crude sheep pituitary extract as evidenced by uterine weight changes. The effect may be compared with that of 5 mg. of pituitary extract given alone. There is clearly no indication of inhibitory activity, but quite the contrary there is a slight but consistent augmentation of uterine weights which appears in case of either normal or antihormone tissues irrespective of the organ or animal source.

Popliteal Lymph Node Studies

The results obtained from the study of 3 series of rabbits after the injection of pituitary extract into the sole of the foot are presented

in Table 4. In this table the dose refers to the weight of the extirpated lymph nodes which were homogenized in their entirety for injection. That the treatment had a marked effect on the popliteal lymph nodes of the injected side may be seen by a comparison of the weights with those of the uninjected side.

The data show that injection of pituitary extract into the sole of the foot of rabbits does not cause the formation in the popliteal lymph nodes of demonstrable amounts of the inhibitory substance as uterine weights from experimental animals treated with homo-

TABLE 4. POPLITEAL LYMPH NODES OF RABBITS AND RATS ASSAYED FOR ANTIGONADOTROPHIC ACTIVITY

Autopsy time after injection	Treated popliteal lymph node		Untreated popliteal lymph node		Other lymph nodes		Material injected and dose mg.
	Dose mg.	Ut. wtg. mg.	Dose mg.	Ut. wtg. mg.	Dose mg.	Ut. wtg. mg.	
Series 1—Single injection							
4 days	582	50 (2)*	113	53 (2)	513	73 (1)	F119†
6 days	365	41 (2)	69	53 (2)	168	37 (1)	
8 days	397	43 (2)	201	52 (2)	427	70 (1)	1000 mg.
Control = 40 (5)							
Series 2—Single injection							
4 days	189	53 (1)	—	—	189	45 (1)	ISAP‡
6 days	522	51 (1)	—	—	522	74 (1)	
8 days	180	35 (1)	—	—	180	51 (1)	250 mg.
Control = 52 (3)							
Series 3—Double Injection							
10 days	450	54 (1)	—	—	450	44 (1)	ISAP
4 days							250 mg. each inj.
Control = 52 (3)							

* Numbers in parentheses indicate number of assay animals.

† A purified extract of pig pituitary.

‡ A crude sheep pituitary extract.

genates of these organs are equal to or even greater than those from the control animals which received only pituitary extract. A similar study using rats gave the same general results as those for rabbits shown in Table 4.

Adrenal Cortical Extract

Three examples of the failure of adrenal cortical extract to produce any consistent alteration in the blood serum antigenadotrophic potency of antihormone rats are given in Figure 1. For this study the pooled serums of immunized animals were assayed in various doses at intervals after injection of cortical material and compared with antigenadotrophic serum from the same animals withdrawn before cortical treatment. The representative curves were drawn by

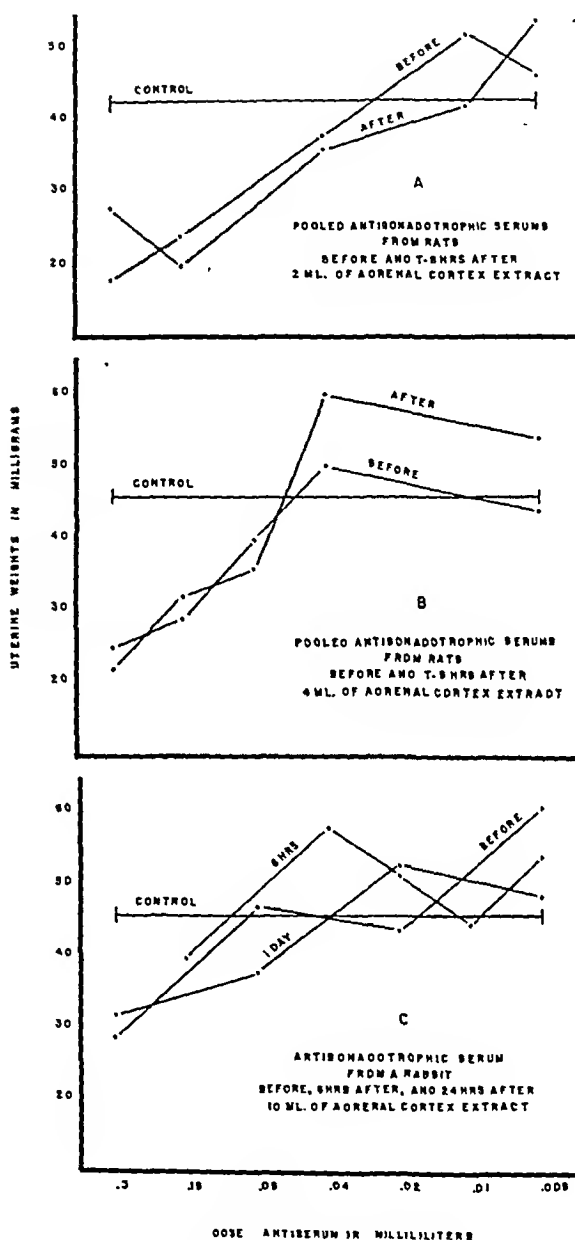


FIG. 1. Negative effect of adrenal cortical extract on the antigonadotrophic titer of blood serum from animals protractedly treated with crude sheep pituitary extract.

plotting uterine weights against decreasing doses of serum. Each point on the graph and the control lines represent the mean uterine weights of 3 assay animals. Figure 1, A and B, compares the pooled antisera of rats before and after subcutaneous injection of 2 and 4

ml. of adrenal cortical extract. Figure 1, C, compares the serum taken from a rabbit before, 6 hours and 24 hours after the injection of 10 ml. of the same extract. The average total drop of the lymphocyte count for the 2 series of rats was marked but not profound. No blood cell counts were made of the treated rabbit.

Doses of .3-.08 ml. of antiserum produced average uterine weights which were quite comparable before and after treatment with adrenal cortex extract indicating that no important change had taken place in the antigonadotrophic titer. The results obtained with smaller doses of antiserum were less consistent and more difficult to interpret although there is a tendency for uterine weights to gradually increase even to a level above that for controls at some point on the curve. As has been pointed out this is the usual response to small doses of dilute antigonadotrophic serum.

Lymph Node Antiserum

The lymph node antiserum proved to be remarkably effective in reducing the number of circulating lymphocytes. One animal given .1 ml. intracardially showed a drop in circulating lymphocytes of 68% after 10 minutes. Another showed a decrease of 73% 5 hours after injection of .05 ml. of the antiserum. But in neither case was there any indication of alteration of the concentration of inhibitory substances in the blood serum of these animals.

CONCLUSION

In the introduction literature was cited which reviews the data supporting the theory that lymphoid tissue, or even the lymphocyte, may be the source of antibody, the rate of release of which is controlled by the pituitary gland through its effect upon the secretions of the adrenal cortex. Data obtained more recently, however, do not support this point of view. Complete summaries of such evidence and additional findings have been presented by Valentine, Craddock, and Lawrence (1948) and Craddock, Valentine, and Lawrence (1949). And other contradictory indications may be found in reports by Westwater (1940), de Gara and Angevine (1943), Phillips, Hopkins, and Freeman (1947), Murphy and Sturm (1947), Eisen *et al.* (1947), Stoerk, John, and Eisen (1947), Thatcher, Houghton, and Ziegler (1948), and Craddock and Lawrence (1948).

From the present report it must be concluded that, in so far as the antigonadotrophic substance may be considered to be an antibody, the findings presented do not support the contention that lymphoid tissue is the source of antibody the rate of release of which is controlled by the secretions of the adrenal cortex, since it was not possible to demonstrate the antigonadotrophic substance in lymphoid tissues, nor was it possible to alter the antigonadotrophic titer of the blood serum of immune animals by adrenal cortical extract or lymph node antiserum.

SUMMARY

Lymphocytes collected by cannulation of the thoracic duct of rabbits and homogenates of lymph node, spleen, liver, bone marrow, cecum, and thymus from rats and rabbits treated for prolonged periods with crude sheep pituitary extract gave no evidence of antigenadotrophic activity. Lymph plasma showed almost the same antigenadotrophic titer as the blood serum.

Injections of pituitary extract into the sole of the foot of rats and rabbits did not result in demonstrable amounts of antigenadotrophic substance in homogenates of popliteal and iliac lymph nodes.

Adrenal cortical extract and lymph node antiserum did not alter the antigenadotrophic titer of blood serum of immunized animals.

Based on the assumption that the antigenadotrophic substance is an antibody, the conclusion is drawn that these findings do not support the view which regards lymphoid tissue in general and the lymphocyte in particular as the source of antibody the release of which is brought about by adrenal cortical substance.

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INDUCTION OF PSYCHIC ESTRUS IN THE HAMSTER WITH DESOXYCORTICOSTERONE ACETATE AND ITS EFFECTS ON THE EPITHELIUM OF THE LOWER REPRODUCTIVE TRACT

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KLEIN (1937), Frank and Fraps (1945) and Kent and Liberman (1947, 1949) have shown that ovariectomized golden hamsters will mate after treatment with estrone and progesterone. Miescher, *et al.* (1938) and Wells and Greene (1939) described progestational changes in the adrenalectomized rabbit uterus after estrone-desoxycorticosterone administration. Van Heuverswyn, *et al.* (1939) described the uterine progestational changes induced with desoxycorticosterone in adrenalectomized rabbits and guinea pigs, and concluded that this substance was 1/6 to 1/10 as potent as progesterone in eliciting these changes. These investigators obtained psychic estrus in 6 of the 10 animals so treated. Other investigators, as Sosa Gallardo (1939), Courrier (1940), Leathem and Crafts (1940), Masson and Selye (1942), Masson (1943) and Emery (1946), have reported inducing progestational changes using desoxycorticosterone.

The purpose of this paper is twofold: first, to determine the effectiveness of desoxycorticosterone acetate (DCA) in inducing psychic estrus in the ovariectomized golden hamster primed with estrone; and, second, to ascertain the nature of DCA-induced epithelial changes in the lower reproductive tract of the hamster.

MATERIALS AND METHODS

Twenty-two normal, mature, virgin female golden hamsters (*CRICETUS AURATUS*) of the LSU strain were used in the present study. Only those animals weighing a minimum of 85 grams and exhibiting typical estrous cycles (as determined by the vaginal smear technique) prior to ovariectomy, were used in this study.

All animals were bilaterally ovariectomized using a mid-ventral incision after anesthetization with sodium pentobarbital. A recuperatory period of 21 days intervened before hormone treatment was begun. During the last 8 days of this period the vaginal smears were observed daily in order to ascertain whether or not smears might be of the castrate type, and animals were paired nightly to be certain that mating would not occur.

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¹ Contribution No. 97. This paper is based on research performed in partial fulfillment of the requirement for the degree of Master of Science under the direction of George C. Kent, Jr.

During the experiment 0.05 mg. estrone² diluted in neutralized olive oil was administered subcutaneously in the ventral abdominal wall for 6 consecutive nights in 43 trials. The final dose was followed in 24 hours by 1.0 mg. desoxycorticosterone acetate.³ The estrone regimen is of the same magnitude as was used by Van Heurverswyn *et al.* (1939), in studies on the guinea pig, and by Kent and Liberman (1947, 1949) in their studies of the effects of estrone and progesterone on vaginal smears in the hamster. During the entire regimen the animals were tested nightly for mating responses, and vaginal smears were observed at the same time. In view of the observations of Kent and Liberman (1949) (who noted that the female hamster did not mate during 4 hours of observation after the administration of estrone even when combined with a very small amount of progesterone), it was considered unnecessary to test the mating reaction of estrone-injected hamsters for longer than 30 minutes. Those animals which mated during the estrone regimen, 7 instances in 43 trials, were discarded. After the administration of DCA, the animals were observed every half hour until the female exhibited lordosis and copulation occurred. Following a complete regimen of hormones, the animals were allowed to rest for a minimum of 14 days before they were used for repeating the experiment. During the final trial, groups of animals were killed 24 hours after the complete estrone regimen, and 24 and 48 hours after DCA administration. The reproductive tract of each animal was removed. The upper vagina, cervix, body and horn of the uterus (as defined by Comeaux, 1949) were utilized in the present study. These tissues were fixed in Bouin's fixative (PFA) and stained with Delafield's hematoxylin, eosin being used as a counterstain.

RESULTS

In 16% of the trials, mating occurred with estrone alone. The total quantity of estrone that had been administered varied from 0.2 mg. to 0.3 mg. This percentage is in accord with the findings of Kent and Liberman (1947). In all 36 trials on 18 animals receiving the DCA following estrone psychic estrus was exhibited. However, the time interval necessary for the exhibition of psychic estrus following DCA varied from 51 minutes (1 animal) to 115 minutes (1 animal), the average time being 87 minutes.

The epithelia of the vagina, cervix, body and horn of the uterus were studied in order to determine whether or not noticeable proliferation had occurred. No actual measurements of the extent of proliferation were made. That definite proliferation occurred may be verified in the accompanying figures. Twenty-four hours after the final estrone injection, the epithelium of the upper vagina and cervix was found to be composed of cuboid cells beneath a thick surface layer of squamous cells, which latter were undergoing extensive desquamation (figs. 1, 4). Twenty-four hours after the administration of DCA a definite thickening of the epithelium had taken place. Desquamation had ceased and the epithelium was composed of a thin surface layer

² Theelin-in-Oil.

³ Percorten.

of squamous cells overlying many large cuboid cells (figs. 2, 5). Forty-eight hours after DCA treatment, the only obvious change observed was in the cell types present, large cuboid cells now occurring on the surface, the underlying cells being smaller and cuboidal. No change was observed in height of the epithelium, but extracellular vacuolation was observed in both regions (figs. 3, 6).

The epithelium of the body of the uterus, 24 hours after estrone treatment (fig. 7), was similar to that of the cervix. Twenty-four hours after the administration of DCA (fig. 8), there was little noticeable change in the epithelium of the uterine body either with regard to thickness or cell types present. The most noticeable change was the cessation of desquamation. Definite thickening of the epithelium and increased vacuolation was clearly observed after 48 hours had elapsed. At this time only large cuboid cells were present (fig. 9).

The epithelium of the horn of the uterus underwent definite progestational proliferation after treatment with DCA. Twenty-four hours after the complete estrone regimen the uterine horn epithelium was composed typically of pseudostratified columnar cells (fig. 10). Twenty-four hours after administration of DCA, the epithelial cells had become larger and resembled large cuboid cells. The basement membrane had become obscure and some vacuolation of the endometrium was evident (fig. 11). Forty-eight hours after DCA treatment the endometrium showed little alteration. The surface of the epithelium was highly pitted, unlike the condition seen at any other time. Vacuolation had not appreciably increased. The cells of the epithelium were, for the most part, cuboidal (fig. 12).

DISCUSSION

In the present experiments utilizing the hamster, all the animals treated with estrone and desoxycorticosterone acetate mated. The time interval (an average of 87 minutes) was shorter in the hamster than the 7.3 hours Van Heuverswyn reported for the guinea pig. The latent period between the injection of DCA and the exhibition of psychic estrus in the present experiment appears about the same as that observed by Kent and Liberman following progesterone. From a consideration of the cyclic changes in the different regions of the normal reproductive tract as described by Comeaux one would infer that, if any progestational changes were to be produced by DCA, the vagina and cervix would be most likely to be affected. Those are the

All figures are cross sections at a magnification of $\times 150$.

FIG. 1. Upper vagina 24 hours after estrone.

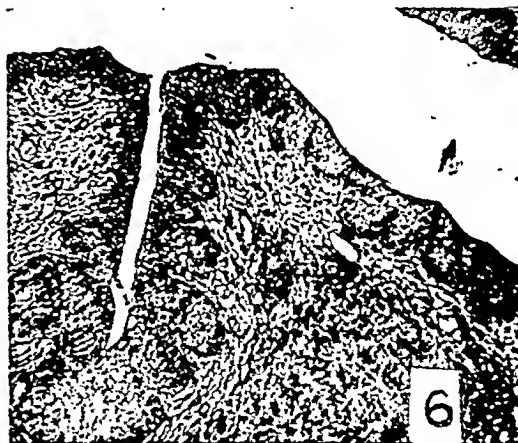
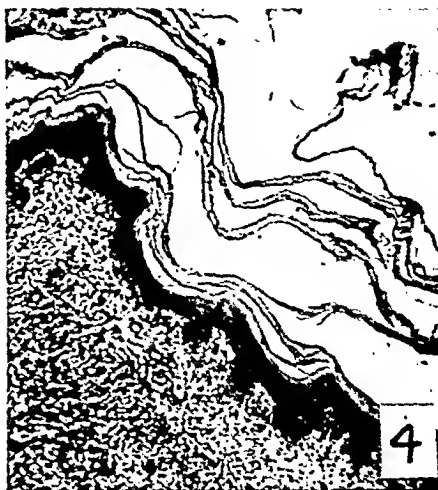
FIG. 2. Upper vagina 24 hours after estrone-DCA.

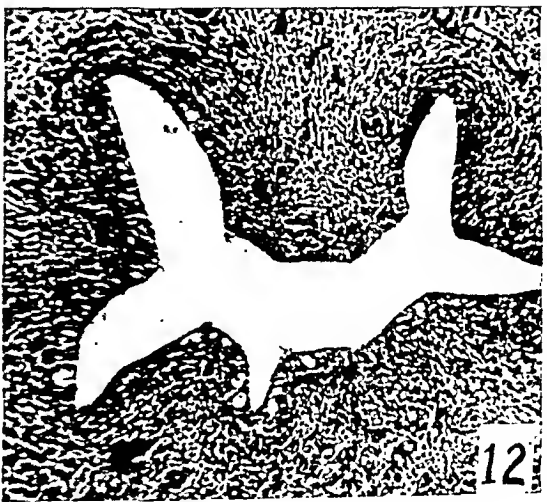
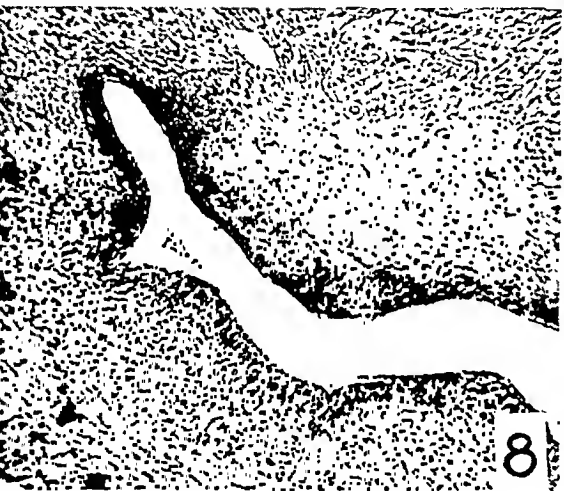
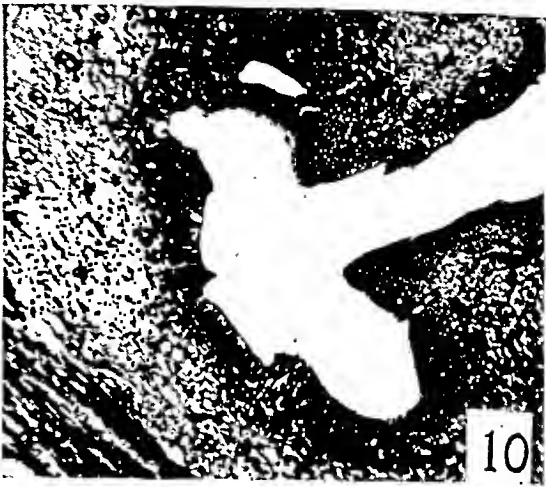
FIG. 3. Upper vagina 48 hours after estrone-DCA.

FIG. 4. Cervix 24 hours after estrone.

FIG. 5. Cervix 24 hours after estrone-DCA. Upper portion of figure cervix, lower portion of figure upper vagina.

FIG. 6. Cervix 48 hours after estrone-DCA.





regions where most of the progestational changes actually occurred. White (1949) found that the epithelium of the uterine horn of intact animals was not altered during pregnancy, pseudopregnancy, or during the reproductive cycle in nulliparous animals. In the present experiments utilizing DCA, not only did the upper vagina and cervix exhibit proliferation, but the body and horn of the uterus likewise showed proliferative changes.

There exists the possibility that substances produced by the intact adrenal glands of these animals may have affected the results, since the animals were not adrenalectomized.

SUMMARY

Psychic estrus was induced in ovariectomized golden hamsters in each of 36 tests using a regimen of 0.05 mg. estrone for 6 nights followed in 24 hours by 1.0 mg. desoxycorticosterone acetate. An average interval of 87 minutes elapsed after desoxycorticosterone acetate was administered and before psychic estrus ensued.

Proliferation occurred in the epithelium of the upper vagina following estrone-desoxycorticosterone acetate injections. Proliferation was most pronounced 24 hours after the hormone treatment. At this time the epithelium of the cervix was thickened. An increase in the size of the epithelium in the body of the uterus was noted after 48 hours, but was not comparable in extent to that in the cervix. After 24 hours, the cells of the epithelium of the uterine horn also exhibited pronounced enlargement.

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- FIG. 7. Uterine body 24 hours after estrone.
FIG. 8. Uterine body 24 hours after estrone-DCA.
FIG. 9. Uterine body 48 hours after estrone-DCA.
FIG. 10. Uterine horn 24 hours after estrone.
FIG. 11. Uterine horn 24 hours after estrone-DCA.
FIG. 12. Uterine horn 48 hours after estrone-DCA.

THE EFFECTS OF 17-VINYL TESTOSTERONE UPON THE RAT ADRENAL¹

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INTRODUCTION

TEPPERMAN, Engel and Long (1943) have reviewed the literature concerning adrenal hypertrophy and have pointed out the factors which lead to cortical atrophy. Clinical studies by Albright, Reifenshtein and Forbes (1946) have shown that the administration of methyl testosterone reduces the urinary excretion of 17-ketosteroids and 11-oxysteroids in normal women and patients with adrenogenital syndrome. They pointed out the desirability of obtaining a steroid which would "inhibit the androgenous source without itself being an androgen."

The ultimate purpose of this investigation was to find a substance which might in such a manner control congenital hyperplasia of the adrenal in females. The effect of 17-vinyl testosterone and of other androgenic and non-androgenic steroids was investigated.

Studies were made of the weight and cholesterol concentration of the adrenals of male and female rats treated with these steroids. In addition, the role of the gonads and the pituitary was studied by administering the compounds to gonadectomized and hypophysectomized animals.

METHODS AND MATERIALS

Female albino rats were selected for most of these experiments, although males were also used in certain special cases. The animals were maintained on a diet of Purina Dog Chow and fresh vegetables. The rats were autopsied at 80 to 84 days of age, at which time the average body weight was approximately 180 grams. The organs were weighed on a torsion balance. Organ weights were calculated on the basis of milligrams per 100 grams of body

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² Eli Lilly Fellow in Medicine.

³ Argentine Fellow of the Mead Johnson Fellowship of the Society for Pediatric Research.

weight. One adrenal, the largest of the pair, was placed in Bloor's solution, ground and extracted. The cholesterol concentration of the pooled extract from each group of animals was determined by the method described by Peters and Van Slyke (1932). The other adrenal gland from each animal was placed in 15 cc. of 6% trichloroacetic acid and ground with acid washed norit. The ascorbic acid concentration of the pooled extract from each group of animals was determined by the method of Roe and Kuether (1943). The steroids injected were dissolved in sesame oil with additional benzyl alcohol when necessary. Gonadectomized and hypophysectomized animals were operated on immediately prior to the injection of steroid hormones.

TABLE 1. THE EFFECT OF DIFFERENT DOSES OF METHYL TESTOSTERONE IN FEMALE RATS

Number of animals	Daily dose mg	Number of days	Average daily weight gain gm.	Average adrenal weight mg. per 100 gm. Range given in parenthesis	Average adrenal cholesterol mg. per 100 mg. adrenal tissue
4	0	0	2.3	17.9 (14.7-21.1)	.306
4	2.5	20	2.6	10.8 (9.6-12.9)	.226
4	5	10	2.0	14.0 (12.0-15.7)	.222
4	5	20	2.2	13.6 (11.5-18.4)	.142
3	5	40	2.1	13.9 (10.9-17.5)	.149
4	10	20	2.1	16.2 (14.3-19.0)	.154

TABLE 2. THE EFFECT OF DIFFERENT DOSES OF DESOXYCORTICOSTERONE ACETATE IN FEMALE RATS

Number of animals	Daily dose mg.	Number of days	Average daily weight gain gm.	Average adrenal weight mg. per 100 gm. Range given in parenthesis	Average adrenal cholesterol mg. per 100 mg. adrenal tissue
4	0	0	2.4	18.9 (16.9-21.3)	.346
3	(Oil)	40	2.7	18.8 (16.9-21.3)	.418
4	1.25	20	2.6	18.0 (16.1-20.6)	.346
4	2.5	10	2.3	18.9 (14.5-24.1)	.466
4	2.5	20	2.1	17.9 (15.6-20.3)	.404
3	2.5	40	2.2	16.8 (16.7-17.0)	.384
4	5.0	20	2.2	13.1 (10.8-16.6)	.410

RESULTS

The effect of various doses of steroid upon the adrenal size and cholesterol concentration is illustrated in Tables 1, 2, 3 and 4, in which are shown the results when methyl testosterone,* desoxycorticosterone,* progesterone and 17-vinyl testosterone* were given to young females. A definite reduction in adrenal weight was produced with as little as 2.5 mg. methyl testosterone, 5 mg. of desoxycorticosterone,

* Graciously furnished by Dr. E. Oppenheimer, Ciba Pharmaceutical Products, Inc.

5 mg. of progesterone and 2.5 mg. of 17-vinyl testosterone given for 20 days. It is important to note that higher doses of methyl testosterone caused less reduction in adrenal weight than the smaller doses, while with desoxycorticosterone, progesterone and 17-vinyl testosterone the higher the dose of administered steroid the greater the reduction in adrenal weight. In those cases where the period of treat-

TABLE 3. THE EFFECT OF DIFFERENT DOSES OF PROGESTERONE IN FEMALE RATS

Number of animals	Daily dose mg.	Number of days	Average daily weight gain gm.	Average adrenal weight mg. per 100 gm. Range given in parenthesis	Average adrenal cholesterol mg. per 100 mg. adrenal tissue
4	0	0	2.2	19.7 (17.0-24.9)	.374
4	(Oil)	20	2.2	18.9 (13.5-23.8)	.353
4	1.25	20	2.3	18.1 (15.5-21.3)	.355
4	2.5	10	2.4	17.6 (12.1-21.8)	.367
4	2.5	20	2.4	18.6 (13.8-22.3)	.296
3	2.5	40	2.2	17.7 (18.5-19.2)	.429
4	5.0	20	2.1	14.8 (13.0-16.1)	.428

TABLE 4. THE EFFECT OF DIFFERENT DOSES OF 17-VINYL TESTOSTERONE GIVEN FOR 20 DAYS IN FEMALE RATS

Number of animals	Daily dose mg.	Average daily weight gain gm.	Average adrenal weight mg. per 100 gm. Range given in parenthesis	Average adrenal ascorbic acid 100 mg. adrenal tissue	Average adrenal cholesterol mg. per 100 mg. adrenal tissue
5	0	1.4	14.8 (8.7-19.8)	.443	.352
5	(Oil)	1.5	15.8 (11.9-20.8)	.526	.356
5	1.25	2.0	13.2 (9.0-18.6)	.547	.390
5	2.50	1.2	10.6 (7.5-13.9)	.582	.428
5	5.00	2.1	9.4 (5.9-13.1)	.566	.408
5	Testost. Propionate 2.50	1.5	14.1 (12.8-15.7)	.536	.179

ment was shortened to 10 days or lengthened to 40 days, the results were similar to those obtained during the 20 day treatment period, although with longer treatment some of the observed changes were more marked.

The cholesterol concentration of the adrenals was markedly reduced when methyl testosterone or testosterone propionate was given and this effect was noticeable at all dosage levels employed. On the other hand, the administration of desoxycorticosterone, progesterone and 17-vinyl testosterone caused no reduction in the adrenal cholesterol concentration and in some instances there was a slight rise. No significant changes in adrenal ascorbic acid were noted. When the

cholesterol was measured as free and ester fractions it was found that both were proportionately reduced.

Table 5 gives the results of a single experiment in which several steroids were given to different groups of animals. While testosterone propionate and 17-vinyl testosterone produced a reduction in adrenal weight, dehydroisoandrosterone acetate, 17-ethynyl testosterone

TABLE 5. THE EFFECT OF 2.5 MG. A DAY OF VARIOUS STEROIDS GIVEN FOR 20 DAYS TO FEMALE RATS

Number of animals	Steroid injected	Average daily weight gain gm.	Average adrenal weight mg. per 100 gm. Range given in parenthesis	Average adrenal cholesterol mg. per 100 mg. adrenal tissue
5	None	2.4	20.7 (14.2-25.1)	.257
6	Testosterone Propionate	2.6	13.1 (11.4-14.6)	.196
5	Dehydroisoandrosterone Acetate	3.0	17.6 (16.4-18.7)	.249
4	Ethynyl Testosterone	2.9	18.7 (18.3-19.8)	.333
4	Vinyl Testosterone	3.1	12.3 (11.0-14.6)	.307
2	Ethyl Testosterone	2.8	18.8 (18.5-19.1)	.374

TABLE 6. THE EFFECT OF 17-VINYL TESTOSTERONE GIVEN FOR 20 DAYS TO NORMAL AND OVARECTOMIZED RATS

Number of animals	Condition of animals	Daily dose mg.	Average daily weight gain gm.	Average adrenal weight mg. per 100 gm. Range given in parenthesis	Average adrenal cholesterol mg. per 100 mg. adrenal tissue
5	Normal	(Oil)	1.5	17.3 (14.2-19.6)	.339
5	Normal	5	2.2	12.5 (9.2-16.0)	.355
6	Ovariectomized	(Oil)	1.0	16.2 (12.9-18.2)	.361
6	Ovariectomized	5	1.9	10.8 (8.8-15.5)	.384

(Figure 2) and 17-ethyl testosterone had little if any effect, although the same dose was used for each compound. Here again testosterone propionate, but none of the other steroids, caused a reduction in adrenal cholesterol concentration. In fact, with desoxycorticosterone, progesterone and 17-vinyl testosterone in large doses there was an increase in the adrenal cholesterol concentration.

Since there seemed to be a degree of parallelism between the reduction in adrenal weight and the atrophy of the ovaries following injections of the steroids, the experiment with 17-vinyl testosterone was repeated with ovariectomized animals (see Table 6). The administration of 5 mg. of 17-vinyl testosterone daily for 20 days produced a 23 per cent reduction in adrenal weight in the normal rats and a 28 per cent reduction in adrenal weight in the gonadectomized rats, although the adrenals were slightly smaller following ovariectomy.

A comparable experiment with normal male and castrate male rats (Table 7) showed a 22 per cent reduction in the weight of the adrenals in normal males and a 20 per cent reduction in the weight of the adrenals in gonadectomized males, although the adrenals were slightly larger following castration.

TABLE 7. THE EFFECT OF 17-VINYL TESTOSTERONE GIVEN FOR 20 DAYS TO NORMAL AND CASTRATED RATS

Number of animals	Condition of animals	Daily dose mg.	Average daily weight gain gm.	Average adrenal weight mg. per 100 gm. Range given in parenthesis	Average adrenal ascorbic acid mg. per 100 mg. adrenal tissue
5	Normal	(Oil)	0.3	12.9 (9.6-17.5)	.409
5	Normal	5.0	0.5	8.9 (8.0- 9.8)	.497
5	Castrate	(Oil)	0.6	13.5 (8.5-17.9)	.406
6	Castrate	5.0	0.0	9.6 (8.8-10.7)	.446

Finally, 17-vinyl testosterone and testosterone propionate were given to hypophysectomized female rats for 20 days in a dosage of 2.5 mg. daily (Table 8). Although the number of animals used was too small to permit a quantitative comparison both 17-vinyl testosterone

TABLE 8. THE EFFECT OF 2.5 MG. OF 17-VINYL TESTOSTERONE AND TESTOSTERONE PROPIONATE INJECTED DAILY FOR 20 DAYS IN HYPOPHYSECTOMIZED FEMALE RATS

Number of animals	Steroid injected	Average daily weight gain gm.	Average adrenal weight mg. per 100 gm. Range given in parenthesis	Average adrenal cholesterol mg. per 100 mg. adrenal tissue
4	None	-0.6	8.95 (7.59-11.65)	.867
3	Testosterone Propionate	-1.3	7.29 (6.25- 8.11)	.721
3	17-Vinyl Testosterone	-1.0	5.79 (4.48- 7.41)	.722

and testosterone propionate treated animals showed smaller adrenals than the untreated controls. The higher than normal adrenal cholesterol concentration which followed hypophysectomy was reduced but not to normal levels by the administration of these steroids.

DISCUSSION

Results obtained with the potent androgenic steroids, testosterone propionate and methyl testosterone, confirm those previously reported and verify the observations made by Selye (1941) that small doses produce greater atrophy than larger doses. Although Hall and

Korenchevsky (1938) noted a decrease in the number of fat vacuoles and lipoid granules in the adrenal cortex after giving androgen, there have been no measurements of the adrenal cholesterol concentration. The finding of a reduced cholesterol concentration in the adrenal gland which has atrophied after treatment with testosterone or methyl testosterone contrasts with the observations of Sayers (1948) that the cholesterol concentration of the adrenal is greater than normal after hypophysectomy. It appears that the cholesterol concentration rises when the reduction in size is brought on by a diminution in pituitary tropic hormone, but falls when the reduction in size is brought on by the administration of androgen.

The decrease in adrenal weight brought on by administering desoxycorticosterone has been reported in detail by Greep and Deans (1947) while the decrease in weight produced by progesterone has been noted by Clausen (1940). There has been no mention in the literature of the effect of 17-vinyl testosterone upon adrenal size. This compound is not generally considered to be androgenic, although it was shown by Kochakian (1944) to have weak properties of this nature. Its effect upon the adrenal gland differed from that of the potent androgens in that large doses had more effect than small doses, and in that there was no reduction in the cholesterol concentration.

The fact that 17-vinyl testosterone produced a reduction in the size of the adrenal glands in ovariectomized females and castrate males, as well as in normal rats of both sexes, indicates that the effect was not mediated by way of the gonads. Results with the hypophysectomized animals are more difficult to interpret since in these animals the adrenal is reduced in size and has a higher than normal cholesterol concentration. Since 17-vinyl testosterone can cause a reduction in the size of the adrenal in the absence of the gonads it seems most probable that this is the result of either a direct effect upon the adrenal gland or an indirect effect through the pituitary or both, and it is of special significance because the compound is not strongly androgenic.

It is of interest to compare the effects of 17-ethyl, 17-vinyl and 17-ethynyl testosterone. These compounds differ only in the degree of saturation of the $C_{20}-C_{21}$ bond. With 17-ethyl testosterone which is known to be relatively inert, Kochakian (1944) did not produce any reduction in adrenal size. The 17-vinyl compound, which has a single unsaturated or ethylene type of bond and which is a very weak androgen (Selye, 1942), caused considerable decrease in the size of the adrenals. The 17-ethynyl compound, which has a double unsaturated or acetylene type of bond, is a strongly progestational compound (Emmens and Parkes, 1939), but it did not produce any decrease in the size of the adrenals.

SUMMARY

Desoxycorticosterone acetate and progesterone in a dosage of 5 mg. daily and testosterone propionate, methyl testosterone and 17-vinyl testosterone in a dosage of 2.5 mg. daily for 20 days, caused a significant decrease in the adrenal weight of female rats.

Testosterone propionate and 17-methyl testosterone caused a decrease in the cholesterol concentration, while progesterone, desoxycorticosterone acetate and 17-vinyl testosterone in large doses caused a slight rise in the cholesterol concentration and hypophysectomy caused a marked increase in the cholesterol concentration of the adrenals.

Although the adrenal atrophy produced by 17-vinyl testosterone was more striking in females than males, the effect was not diminished by ovariectomy or orchidectomy.

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THE DEVELOPMENT OF A REFRACTORY STATE TO ADRENOCORTICOTROPHIC HORMONE¹

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IN VIEW of current interest in adrenocorticotrophic hormone (ACTH) and its widespread experimental use in the clinic and laboratory, it seemed pertinent to determine whether animals remain responsive or become refractory to repeated injections of this substance.

Interest in the development of "antihormones" dates to the work of Collip and Anderson (1934). It is now well-known that the injection of pituitary thyrotrophic hormone or the gonadotrophic hormones of the pituitary or of the chorionic tissues readily induces in animals of heterologous species a refractory state associated with demonstrable antihormones in the serum. Similar, though less well-established, evidence has been presented concerning almost every principle elaborated by the anterior pituitary.

Although the nature of the antihormones has not been definitely established, most of the evidence suggests that they behave, for the most part, as antibodies. Thus, it has been shown that (1) certain of the trophic hormones of the pituitary and chorion call forth complement-fixing and precipitating antibodies (Van den Ende, 1939) as well as antihormones and may be considered true antigens; (2) denaturation may abolish the physiologic effect of the hormone but leave the antigenicity relatively intact (Bischoff, 1948); (3) rate of loss of antihormone activity *in vivo* (Chance, 1940) as well as time relationships of hormone-antihormone union (Wolfe, Meyer and McShan, 1945) are typical of antibody; (4) antihormones are not called forth by homologous extracts (Smith, 1930) nor do they appear in parabiotic animals with prolonged pituitary hyperactivity (DuShane, Levine, Pfeiffer and Witschi, 1935); (5) the site of production of antihormones as well as antibodies is the reticulo-endothelial system (Gordon, Kleinberg and Charipper, 1939); (6) the changes in antihormone species-specificity are compatible with immunological concepts; and (7) the antihormones reside in the globulin fraction of the serum (Harrington and Rowlands, 1937) and have a humoral action, no endocrine organ playing a necessary rôle (Bachman, Collip and

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Selye, 1934). Those investigators who believe that antihormones are *not* true antibodies point to the fact that precipitin titer is often not correlated with serum antihormone activity (Meyer and Gustus, 1935; Meyer and Wolfe, 1939; Gustus, Meyer and Dingle, 1935); they suggest that the antibodies demonstrable *in vitro* are due to impurities in the antigen preparation. Certain non-protein hormones have given rise to antihormones (Hartman, Lewis and Gabriel, 1940); these probably act as haptenes, and are therefore not strictly comparable. The review of Thompson (1941) summarizes in detail the literature to that date.

Until recently, the hormone preparations used as antigens have of necessity been impure to varying degree. The use of the more crude extracts is associated with increased antihormone-producing power in some studies (Rowlands and Young, 1939; Leathem and Abarbanel, 1943) and with decreased power in others (Gordon, Levenstein and Charipper, 1940; McShan, Wolfe and Meyer, 1943). The advantages of studying a pure and homogeneous trophic hormone of the pituitary are obvious.

Sayers, Sayers, Liang and Long (1946) have shown that the administration of ACTH to the rat is followed by a temporary decrease in the adrenal ascorbic acid and cholesterol. There is suggestive evidence that the cholesterol and ascorbic acid depletion are temporally related to elaboration and release of steroid hormones from the adrenal cortex (Long, 1947). Under suitable conditions, the degree of adrenal ascorbic acid depletion, maximal and constant at 1 to 3 hours, is a function of the dose of ACTH administered (Sayers, Sayers and Woodbury, 1948). In the present study, ACTH physiologic activity is measured in terms of adrenal ascorbic acid depletion.

The purpose of the present study was to investigate (1) the response of the rat adrenal to long-term ACTH injection and (2) the development of an antihormone in the serum of the injected rat, demonstrable *in vivo* and *in vitro*.

MATERIALS AND METHODS

Animals. Sprague-Dawley male rats, 28 days of age, were used for the ACTH injections after a one week observation period; poor weight-gainers were discarded. Hypophysectomized male rats² of the Sprague-Dawley strain were used to study the biological activity of the antihormone to ACTH. They weighed 140 to 160 gm. at the time of hypophysectomy (done by the parapharyngeal approach). Completeness of hypophysectomy was judged by atrophy of the gonads, failure to gain weight, and direct examination of the sella at the time of autopsy. All animals were maintained at an environmental temperature of 77°F. throughout, and were fed Purina Laboratory Chow and water ad libitum.

Hormone Preparation. ACTH preparation 41-L-3 was prepared by the

² Some of the hypophysectomies were done by Dr. Jane A. Russell.

Armour Laboratories³ from hog pituitary glands by a modification of the method of Sayers, White and Long (1943); the method precludes appreciable contamination with thyrotrophic, gonadotrophic or growth hormone. This preparation was said to have an ACTH activity of 35% of the Armour Standard Preparation La-1-A⁴, an oxytocic activity of 0.04 units/mg.,⁵ and a pressor activity of 0.04 units/mg.⁶

ACTH powder was stored *in vacuo* over CaCl₂. Every 7 days, sufficient material for the week's injections was dissolved in 0.85% NaCl, neutralized with 0.1 N NaOH. Aliquots of the solution were kept in the frozen state and thawed at room temperature immediately prior to injection.

Protocol of Injection. Group I: At the onset of the experiment, 28 rats received intraperitoneally 1 mg. ACTH (in 0.2 ml.) four times weekly. *Group II:* 24 rats received 0.2 ml. 0.85% NaCl intraperitoneally on the same schedule. At the end of each week, three or four rats from each group were adrenalectomized and sacrificed; thus, the last rats of each group to be adrenalectomized had received injections for 7 weeks. At the end of weeks 1, 2, 4 and 6, four rats from Group I and three rats from Group II were weighed, adrenalectomized, and exsanguinated 24 hours after their last injection. One rat from each group was similarly treated at the end of weeks 3, 5 and 7. At the end of week 3, 24 hours after their last injection, three rats from each group were weighed and given a test injection intraperitoneally of 25 µg. ACTH/100 gm. body weight (in 0.08 to 0.1 ml.); the rats were adrenalectomized and exsanguinated one hour after receiving the injections. At the end of weeks 5 and 7, the week 3 régime was duplicated except that the test dose was 40 µg. ACTH/100 gm. body weight (in 0.15 to 0.25 ml.). *Group III:* Three rats received 0.1 mg. ACTH (in 0.15 ml.) four times weekly for weeks. They were exsanguinated two weeks after the last injection.

Experimental Procedure. At the times indicated, the animals were anesthetized with nembutal intraperitoneally. The left adrenal was removed but left in the peritoneal cavity to prevent drying while the right adrenal was removed; elapsed time between abdominal incision and removal of the right adrenal never exceeded 4 minutes. Each adrenal was transferred to filter paper, dissected free of fat and connective tissue, weighed to the nearest 0.1 mg., and analyzed in duplicate for ascorbic acid by the method of Roe and Keuther (1943) which determines total ascorbic acid. The values for the right and left adrenals were averaged to give the level for each rat; in no case did the difference exceed 35 mg./100 gm. of gland.

Following adrenalectomy the rats were exsanguinated by aortic puncture. The blood was allowed to clot at room temperature and the serum immediately separated by centrifugation. The sera to be used for the *in vitro* antibody tests were refrigerated until needed (120 hours or less). The sera to be used for the *in vivo* antihormone tests were pooled and stored in the frozen state until needed.

Biological Assay Method. Six hypophysectomized rats were given serum

³ The ACTH was obtained through the courtesy of Dr. Edwin E. Hays, The Armour Laboratories, Chicago, Illinois.

⁴ 4 µg. of the reference standard (Armour La-1-A) administered intravenously to hypophysectomized rats induces a 20 to 30 per cent decrease in adrenal ascorbic acid content by the method of Sayers, Sayers and Woodbury (1948).

⁵ Guinea pig uterine strip method.

⁶ Rooster blood pressure method.

from the Group I rats which had received 6 or 7 weeks of ACTH injections; six were given "control" serum from the Group II rats; two were given serum from the Group III rats. Each was injected intraperitoneally as follows: Day 1: 0.25 ml. serum; Day 2: 0.25 ml. serum; Day 3: 0.50 ml. serum. Four to six hours after the last serum injection, each rat received an intraperitoneal injection of 60 μ g. ACTH/100 gm. body weight (in 0.35 to 0.45 ml.) after the removal for assay of the left adrenal. One hour later the right adrenal was removed for assay. Duplicate ascorbic acid determinations on each gland were done as described; response to the test dose was judged by the difference between the left and right adrenals.

Serological Method. Weekly precipitin tests were carried out in which ACTH in solution was used as antigen. Sera from all four treated (Group I) rats and 2 of the control (Group II) rats were tested individually each week; serum from the Group III rats was also tested. When needed, the serum was allowed to thaw at room temperature. 0.3 ml. portions of each serum to be tested were placed in serological test tubes in triplicate. To the first tube 0.1 ml. of 0.85% NaCl containing 0.001 mg. ACTH was added daily for 5 days if no macroscopic precipitate was visible. To the second tube 0.1 ml. of 0.85% NaCl containing 0.01 mg. ACTH was added daily for 2 days if no macroscopic precipitate was visible. To the third tube no antigen was added. (The ACTH concentrations were selected to cover a range from 0.003 to 0.06 mg. antigen per ml. antiserum). All tubes were kept at 4°C. for the duration of the experiment. Tubes were read as negative on the fifth day if no macroscopic precipitate was visible after centrifuging at 1000 R.P.M. for 5 minutes.

RESULTS

1. *The response of the rat adrenal to long-term ACTH injection.*

It is known that following the initial adrenal ascorbic acid depletion after a single ACTH injection, a rebound effect ensues whereby the ascorbic acid level 12 to 24 hours post-injection exceeds the pre-injection level. In Table 1 are summarized the weekly adrenal ascorbic acid values 24 hours after the rats receive their last injection. After one to two weeks of ACTH injections, the "resting" adrenal ascorbic acid level is significantly higher in the rats receiving ACTH. At the end of weeks 4 and 6, the difference is not statistically sig-

TABLE 1. THE EFFECT IN NORMAL RATS OF REPEATED INJECTIONS OF ACTH ON THE ADRENAL ASCORBIC ACID CONTENT 24 HOURS AFTER THE LAST INJECTION

(Averages and standard errors; figures in parentheses represent number of rats)

Week	Adrenal ascorbic acid in mg./100 gm. gland		P value
	Group I (ACTH)	Group II (Saline)	
1	411 \pm 11.6 (4)	373 \pm 13.5 (3)	<0.05
2	484 \pm 10.0 (4)	441 \pm 11.6 (3)	<0.05
3	460 (1)	432 (1)	
4	447 \pm 9.4 (3)	421 \pm 9.4 (3)	>0.10
5	448 (1)	428 (1)	
6	419 \pm 13.0 (4)	411 \pm 15.6 (3)	>0.50
7	430 (1)	430 (1)	

nificant; it is not possible definitely to ascribe this phenomenon to a refractoriness to ACTH, inasmuch as the injected dose of 4 mg. weekly remained constant in rats which were steadily growing. The relatively lower values in each group at the end of one week of injection

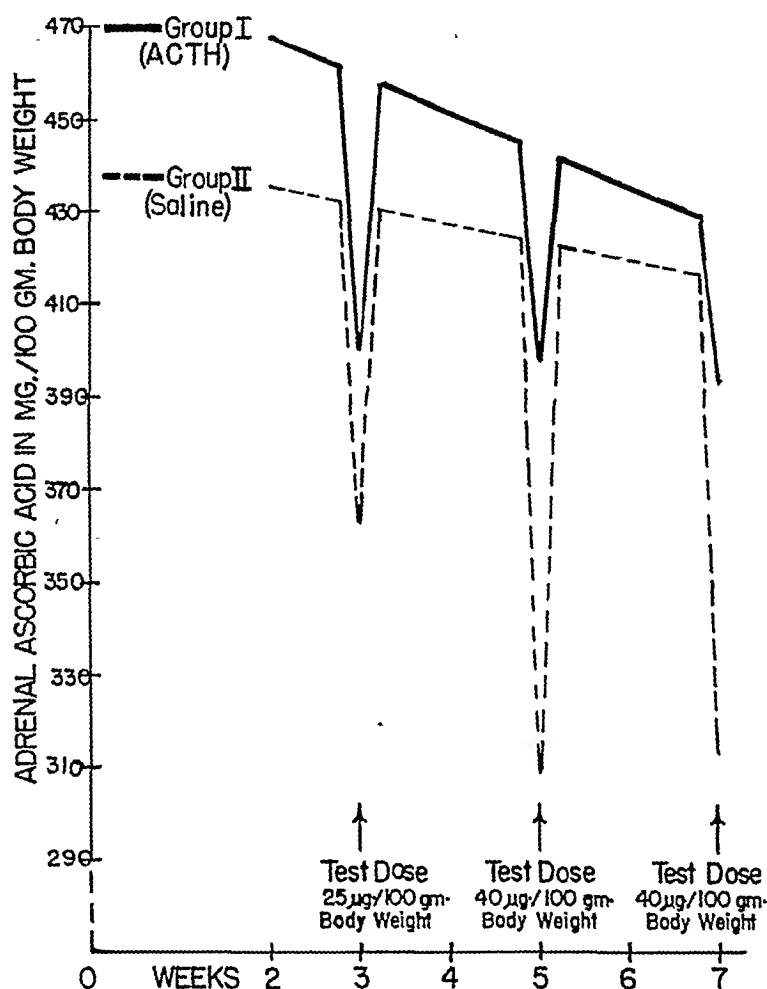


FIG. 1. The effect in normal rats of repeated injections of ACTH on the adrenal ascorbic acid depletion one hour after a test dose of ACTH.

tion may possibly be ascribed to the young age of the rats (40 days) at that time.

The response of the rats to the acute test dose of ACTH at the end of weeks 3, 5 and 7 can best be analyzed if all the "resting" adrenal ascorbic acid values for each group in weeks 2 through 7 be treated as a linear regression, thereby yielding a *calculated* resting value for each group at weeks 3, 5 and 7. This is done in Figure 1. The ACTH-injected rats respond with diminishing vigor to a test

TABLE 2. THE EFFECT IN NORMAL RATS OF REPEATED INJECTIONS OF ACTH ON THE ADRENAL ASCORBIC ACID DEPLETION ONE HOUR AFTER A TEST DOSE OF ACTH

Week	Test dose ACTH	Adrenal ascorbic acid in mg./100 gm. gland							
		Group I—ACTH rats				Group II—Saline rats			
		Resting value*	After test dose†	% decrease	P value	Resting value*	After test dose†	% decrease	P value
3	25 μ g./100 gm. body wt.	460 \pm 10.2	401 \pm 14.9 (3)	13	<0.02	432 \pm 8.5	363 \pm 6.9 (3)	16	<0.01
5	40 μ g./100 gm. body wt.	444 \pm 8.8	398 \pm 2.6 (3)	10	<0.01	424 \pm 7.1	309 \pm 25.4 (3)	27	<0.01
7	40 μ g./100 gm. body wt.	428 \pm 14.9	394 \pm 9.4 (3)	8	>0.20	416 \pm 12.2	313 \pm 13.5 (3)	25	<0.01

* Calculated resting value; standard error derived from variance from regression line.

† Averages and standard errors; figures in parentheses indicate number of rats.

dose of ACTH which consistently depletes the adrenal ascorbic acid of the saline-injected rats. Indeed, the response of the Group I rats to the week 7 test dose must be regarded as negligible since the adrenal ascorbic acid level after the test dose is not significantly lower than the calculated "resting" level for that week (Table 2).

The effects of long-term ACTH injection upon the adrenal weight and body weight of the rat are indicated in Table 3. The adrenals of the Group I (ACTH) rats were consistently heavier than those of the Group II (saline) rats. Even after 6 and 7 weeks of injections, the

TABLE 3. THE EFFECT IN NORMAL RATS OF REPEATED INJECTIONS OF ACTH ON THE ADRENAL WEIGHT AND BODY WEIGHT

(Averages and standard errors; figures in parentheses represent number of rats)

Week	Paired adrenal weights in mg.			Body weight in gm.	
	Group I (ACTH)	Group II (saline)	P value	Group I (ACTH)	Group II (saline)
1	25.1 ± 0.7 (4)	22.2 ± 0.8 (3)	<0.05	130 ± 2.7 (4)	137 ± 3.1 (3)
2	27.2 ± 0.8 (4)	22.5 ± 0.9 (3)	<0.01	163 ± 2.3 (4)	155 ± 2.7 (3)
3	32.9 ± 1.9 (4)	26.4 ± 1.9 (4)	<0.05	193 ± 2.2 (4)	196 ± 2.2 (4)
4	36.3 ± 1.7 (3)	31.6 ± 1.7 (3)	0.11	236 ± 7.0 (3)	237 ± 7.0 (3)
5	36.6 ± 1.4 (4)	32.4 ± 1.4 (4)	0.08	230 ± 5.8 (4)	249 ± 5.8 (4)
6	38.1 ± 1.1 (4)	31.0 ± 1.2 (3)	<0.01	283 ± 7.4 (4)	269 ± 8.5 (3)
7	44.7 ± 1.2 (4)	36.0 ± 1.2 (4)	<0.01	278 ± 10.4 (4)	284 ± 10.4 (4)

difference is highly significant. One notes that the effect of long-term ACTH injection on adrenal ascorbic acid concentration (Table 1) differs from its effect on adrenal weight. White (1944) has indicated that the quantity of ACTH causing a perceptible increase in the adrenal weight of the hypophysectomized rat is less than that required to maintain a normal concentration of adrenal cholesterol.

There is no consistent difference in body weight between the Group I and Group II rats; for no week is the *p* value statistically significant. This small series therefore fails to corroborate, at the dosage level used, the finding of Evans, Simpson and Li (1943) that pure ACTH inhibits the growth of young male rats.

2. The demonstration of antihormone in the serum of rats receiving long-term ACTH injection.

(a). ACTH-neutralizing properties

Hypophysectomized test rats were given a dose of 60 µg. ACTH/100 gm. body weight. They had previously received injections of serum from rats of Group I, II or III. The results appear in Table 4. The serum from Group I rats consistently diminished the adrenal ascorbic acid depletion normally produced in the hypophysectomized rat by ACTH. The same test dose of ACTH resulted in a marked depletion of adrenal ascorbic acid in hypophysectomized rats pre-treated either with normal serum (Group II) or with serum from animals who had received a small amount of ACTH (Group III).

TABLE 4. THE EFFECT IN HYPOPHYSECTOMIZED RATS OF "ANTI-ACTH" SERUM ON THE ADRENAL ASCORBIC ACID DEPLETION ONE HOUR AFTER A TEST DOSE* OF ACTH.

Rats	Adrenal ascorbic acid in mg./100 gm. gland								
	Pretreatment with group I serum			Pretreatment with group II serum			Pretreatment with group III serum		
	Left	Right	Difference	Left	Right	Difference	Left	Right	Difference
1 through 6†	469	366	103 (22%)	422	214	208 (50%)	495	270	225 (46%)
	434	313	121 (28%)	461	261	200 (44%)	439	223	216 (49%)
7 through 14‡	337	283	54 (16%)	322	241	81 (25%)			
	332	272	60 (18%)	362	246	116 (32%)			
	324	275	49 (15%)	347	250	97 (28%)			
	363	316	47 (13%)	353	268	85 (24%)			

* 60 µg ACTH/100 gm. body weight.

† Hypophysectomized 3 days previously.

‡ Hypophysectomized 10 days previously.

(b). *Precipitin-producing properties*

Attempts to demonstrate precipitating antibodies *in vitro* with the technique described were uniformly unsuccessful. A possible explanation is the fact that the Group I rats were sacrificed either 24 hours or 1 hour following an injection of ACTH. It is known that a "negative phase" follows the administration of antigen or hormone, during which period demonstrable antibody or antihormone diminishes markedly; in the studies of Wolfe, Meyer and McShan (1945), the negative phase for antigonadotrophin persisted for about 48 hours. That the hormone-neutralizing effect was more easily demonstrable than the precipitin-producing effect in the present study is in general agreement with the findings of most other workers.

Although the Group III rats were sacrificed at a time when the negative phase was not a factor, they received only 2.8 mg. ACTH *in toto*. That they failed to develop either hormone-neutralizing antihormone or precipitating antihormone indicates that the hormone dose employed in this group was too low to be effective.

DISCUSSION

The data pertaining to refractoriness to adrenocorticotrophic hormone are scant. Thompson and Cushing (1934) postulate its presence in one of their puppies, though the extract used was a crude one. Anderson, Page and Li (1947) found that continued ACTH injection re-elevated only temporarily the post-hypophysectomy blood pressure of rats with renal hypertension. During the preparation of this manuscript, Chase (1949) reported that pure ACTH produced in mice an antihormone which prevented the normal ACTH-induced increase in adrenal weight in the rat and which could be demonstrated serologically by both precipitin and collodion particle agglutinin techniques; the serological activity of the antiserum could be correlated with its biological activity. Larger quantities of ACTH than in the present study were used by Chase, both *in vitro* and *in vivo*,

perhaps accounting for her successful demonstration of precipitating antibodies.

In a recent report, Hensch, Kendall, Slocumb and Polley (1949) note that the striking remission in the signs and symptoms of rheumatoid arthritis produced by Compound E or ACTH requires the continued administration of either hormone. The clinical implications of the development of an antihormone to ACTH are obvious. Long-term therapy may become decreasingly effective, as is the case with some of the gonadotrophic preparations used clinically (Leathem, 1944).

The rats in the present study became refractory to ACTH while receiving doses which diminished from approximately 10 to 5 mg. of ACTH per kg. of body weight per week (expressed in terms of the Armour Standard). This occurred despite the fact that the rat, particularly the young one (Gordon, Levenstein and Charipper, 1939), does not produce antibody as well as many other laboratory animals, and that antihormone probably forms less readily with intraperitoneal than with subcutaneous hormone injection (Leathem, 1947). A 70 kg. patient, receiving the 100 mg. of ACTH per day recommended by Hensch (1949) for the treatment of rheumatoid arthritis, receives approximately 10 mg. of ACTH per kg. of body weight per week. Recently, Leathem (1949) has found that the serum from a patient who had received less than 1500 mg. ACTH inhibited in the rat the (histologically determined) lipid depletion of the adrenal normally produced by ACTH.

It has long been known that continued injection of pituitary extracts from heterologous species may render animals refractory to their own pituitary hormones, with subsequent atrophy of the "target" glands (Bachman, Collip and Selye, 1934); Severinghaus and Thompson (1939) found that the hypophyses of dogs long refractory to a crude pituitary extract had all the changes characteristic of castration, of thyroidectomy, and possibly of adrenalectomy. Although the question of suppression of endogenous ACTH was not studied in the present relatively short-term experiment, one might speculate as to the possible effect of antihormone on the pituitary-adrenal system of a refractory animal or patient who continues to receive heterologous ACTH.

SUMMARY

Rats receiving repeated injections of purified hog ACTH gradually became refractory to a test dose of the hormone which depleted consistently the adrenal ascorbic acid concentration of control rats. After seven weeks of ACTH injections, their response to the test dose was negligible. Serum from such rats contained a substance which consistently diminished the adrenal ascorbic acid depletion normally produced in the hypophysectomized rat by ACTH. Activity of the

antiserum was not demonstrable *in vitro* by the precipitin technique employed.

On a body weight basis, the dosage level of ACTH producing the refractory state in rats is comparable to that which has been used in man in the treatment of rheumatoid arthritis. Some possible implications of this finding are discussed.

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A NEURAL TIMING FACTOR IN THE MECHANISM BY WHICH PROGESTERONE ADVANCES OVULATION IN THE CYCLIC RAT

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INTRODUCTION

UNDER certain conditions progesterone will advance ovulation in cyclic rats about 24 hours (Everett, 1944a, 1948). This result occurs, not in 4-day cycles, but very regularly in 5-day cycles when progesterone is administered about noon on the third day of diestrus. Various reasons were presented (1948) for concluding that this effect is brought about indirectly through the hypophysis by inducing the release of luteinizing hormone (or total gonadotrophin).¹

Independent observations have been made by Fraps and Dury (1943) that in the domestic hen progesterone administration will advance ovulation several hours. More recent evidence (Rothchild and Fraps, 1949) shows that this process, like that in rats, requires the presence of the hypophysis. Their findings indicate, furthermore, that a direct time relationship exists between the hour of progesterone injection and the hour of response, release of the "ovulating hormone" occurring about 4 hours after the injection of progesterone.

A recent study in this laboratory (Everett, Sawyer and Markee, 1949) demonstrated that in 4-day cyclic rats, in our colony, the ovulating discharge of "LH" is controlled by neurogenic stimulation which reaches the gland between 2 and 4 P.M. on the day of proestrus. Injection of Dibenamine (N, N-dibenzyl- β -chloroethylamine) at 2 P.M. or earlier usually blocked or partially blocked the stimulus for LH release, while injection at 4 P.M. or later rarely interfered. Similarly, atropine injected at 2 P.M. or earlier blocked the stimulus but did not block when injected at 4 P.M.

It was of considerable interest to learn whether a neural factor is involved in the hypophyseal liberation of LH in response to progesterone. This appeared quite likely since Sawyer, Everett and Markee (1949) had demonstrated that LH release in response to estrogen may be blocked by either Dibenamine or atropine. The initial experiments

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¹ There is no certainty that the hormone released is only LH (ICSH). It is, however, simpler to say "LH release" than "release of whatever hypophyseal hormones are essential for ovulation," our full meaning. "LH release," "LH discharge," "LH-release apparatus" and similar expressions will be employed freely in this sense.

presented below demonstrate that these agents will also prevent the response to progesterone.

A second question, and the principal one with which the remaining experiments are concerned, is the question of timing: Does pituitary activation in response to progesterone occur at a uniform interval after injection or is it confined to certain hours of the day independent of the injection time?

MATERIAL AND METHODS

Altogether, 137 adult female rats of the Vanderbilt (Osborne-Mendel) strain are represented in the several experimental series. These were vari-

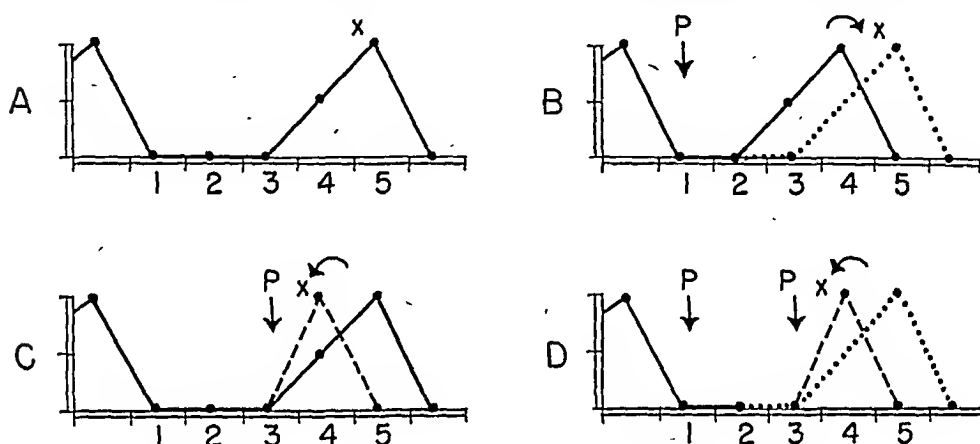


FIG. 1. (Modified from Everett, 1948) A. The standard 5-day cycle, numbered from the first day of diestrus. Proestrus represented by 1 unit on the ordinate, full vaginal estrus by 2 units. Each unit of the abscissa represents 1 day. Ovulation time represented by "X" after midnight following proestrus. B. Artificial 5-day cycle produced in a 4-day cyclic rat by progesterone (p) injected on the first day of diestrus. Dotted line represents the retarded vaginal smear sequence. Ovulation delayed about 24 hours, the shift being indicated by the arrow. C and D. Advancement of ovulation in natural or artificial 5-day cycles, resp., by progesterone injected on diestrus day 3. Arrows indicate the shift in ovulation time caused by this treatment. Broken line shows vaginal smear sequence thus induced.

ously members of the substrains Va and Vc (cf. Everett, 1948). Although most of the former were fundamentally 4-day cyclic animals, the experimental cycle in each such case was transformed to a 5-day cycle by the injection of 2 mg. of progesterone on the first day of diestrus (Everett, 1948) as represented in figure 1, C and D. In one critical experiment to be mentioned later (in Series III) we established that the chronology of events is closely similar in both the natural and artificial 5-day cycle. As the Va stock was more readily available we employed the latter almost exclusively in Series IV.

We have previously described in considerable detail our laboratory routine of determining regularity of cycles by the vaginal smear method (Everett, 1948; Everett, Sawyer and Markee, 1949). Likewise, the day-by-day changes of the vaginal smear characteristic, respectively, of the 4-day and 5-day cycles in our stock have been elucidated elsewhere (Everett, 1948) and will not be repeated here.

The possibility exists that certain errors may result from spontaneous changes in cycle length, occurring in spite of treatment rather than because of it. In the Vc substrain, in which 5-day cycles are the rule, after any 2 such cycles in succession there is an observed 4% frequency of spontaneous shortening of the next cycle to 4 days. This error was considerably reduced by avoiding, with rare exception, the use of animals in which the vaginal leucocyte population was sparse on the morning of "diestrus day 3." In the "artificial" 5-day cycles a similar source of error exists. In a sample of 57 four-day cyclic Vanderbilt rats in which 1.5 to 2.0 mg. progesterone was injected on the first day of diestrus, 4 cases (7%) were encountered in which the treatment failed to prolong the cycle.

The other possibility, that prolongation of the cycle to longer than 5 days might occur, has been observed in 6% of the cases of Vc rats (Everett, 1948). We were able to eliminate this error by excluding as legitimate experiments the few cases in which the smear was leucocytic on the morning of normally expected proestrus. In the artificial 5-day cycles in Va rats 9/82 such instances were encountered and excluded on the same grounds.

The experiments fall into four groups: Series I.—A preliminary series in which either Dibenamine or atropine was administered immediately after, or several hours before, the standard noon injection of progesterone on diestrus day 3. Series II.—A small series in which 5-day cyclic Vc females were given atropine on the day of proestrus at 4 P.M. for comparison with 4-day cyclic rats treated at that hour. Series III.—Injection of progesterone at different hours during diestrus day 3 in 5-day cycles. Series IV.—Injection of progesterone at certain hours, followed at various intervals by the blocking dose of atropine.

Autopsies were performed routinely on the day following experimental treatment, usually in the morning, after 8 o'clock. In the cases (Series III) in which progesterone injection was delayed until 6:30 P.M., autopsy time was delayed until after 10 A.M. Half of these rats, in fact, were autopsied in the early afternoon.

The methods of examining the reproductive tracts and direct observation of tubal ova have been detailed previously (Everett, 1948; Everett, Sawyer and Markee, 1949). Both ovaries were preserved in Zenker's fluid for paraffin imbedding, serial sectioning and staining by our routine procedure.

Progesterone² was prepared from the crystals by dissolving in peanut oil at a concentration of 5 mg./ml. The standard "retarding" dose given on diestrus day 1 (of 4-day cycles) was 2 mg., injected subcutaneously. The standard "ovulating" dose on diestrus day 3 was 1 mg. by the same route.

Dibenamine² was administered as the hydrochloride, prepared according to the method previously described (Sawyer, Everett and Markee, 1949), in Ringer-Locke solution at a concentration of 10 mg./ml. This was injected intravenously, the standard dose being 30 mg./kg. Atropine sulfate (USP XIII, Merck) was made up in Ringer-Locke solution at a concentration of 70 mg./ml., the standard dose being 700 mg./kg. (1 ml./100 gm. body weight). This amount was equally divided between two subcutaneous injection sites.

² Progesterone was generously supplied by the Schering Corporation, Bloomfield, N. J. We are indebted to Dr. William Gump of Glaxo-Delawanna, Inc., Delawanna, N. J., for the Dibenamine hydrochloride.

Atropine was the preferred blocking agent to be used in Series II and IV since Everett, Sawyer and Markee (1949) found that Dibenamine under optimal conditions of timing not infrequently blocks only partially and may even fail entirely. For our present purposes it was especially important to know how rapidly the blocking action of atropine develops after subcutaneous injection. Five adult female rats were given the standard atropine dose and were injected intravenously 10 minutes later with a known lethal dose of a mixture of acetylcholine (1 mg./kg.) and eserine (0.5 mg./kg.). All survived until they were disposed of on the following day. None of the characteristic signs of acetylcholine intoxication was recognized. Therefore, there is little question that whenever this dose of atropine is used, blockade of hypophyseal stimulation will be practically immediate. An error of less than 10 minutes is of no consequence.

The hours of the day as recorded below (and in the allied papers cited above) are in terms of "colony time." Lighting is controlled by a time switch, the length of daily illumination being 14 hours. In terms of Eastern Standard Time the lights are turned on at 6 A.M. and off at 8 P.M. "Colony time" is thus one hour slower than standard, reckoning "midnight" as the mid-point of the dark period.

EXPERIMENTS AND RESULTS

Series I

Preliminary

It was first necessary to determine whether Dibenamine or atropine would prevent the advancement of ovulation by progesterone. Ten 5-day cyclic Vc females were each given progesterone about noon on the third day of diestrus. In 7 of the 10 the progesterone injection was between 11:55 and 12:10 P.M.; Dibenamine was injected 3 to 8 minutes later. At autopsy the next morning none of the animals had ovulated. Histological study of the ovaries revealed no evidence of preovulatory swelling in 2 rats, 1 to 2 follicles in early swelling in each of 4 rats and as many as 5 follicles in early swelling in the 1 remaining rat. In the other 3 of the 10 animals Dibenamine was injected at 8-8:30 A.M., followed by progesterone at 11:50-12:45. None of these rats ovulated overnight; 2 were completely blocked, the other at autopsy had a single follicle in early swelling and a second follicle with a small luteinized plaque. There were thus 4/10 rats in which Dibenamine had completely blocked and 6/10 in which the blocking action was only partially effective.

Two preliminary trials were also made with atropine. Progesterone was given at 12:05 and 12:35 P.M., respectively, followed by atropine within 2 minutes. Neither animal ovulated overnight and histologically the only suggestion of a very minimal effect of LH was the finding of 1 atypical follicle. Mitoses were exceedingly rare and the parietal granulosa was quite disorganized. Although showing a polar body and considerable secretion of secondary liquor about the cumulus, this follicle was judged to be atretic.

These preliminary studies are construed as evidence that the ovulatory discharge of gonadotrophic hormone in response to progesterone requires neurohumoral stimulation.

Series II

Comparison of 5-day cyclic rats with 4-day cyclic rats with respect to critical hours of normal hypophyseal stimulation during proestrus

Our data regarding the normal hours of stimulation of the hypophysis during proestrus (Everett, Sawyer and Markee, 1949) were largely confined to 4-day cyclic rats. As of the present writing, in 4-day cyclic animals the accumulated evidence with various blocking agents indicates 21/27 cases of full stimulation and 4/27 with partial stimulation by 4 P.M. Only 2/27 cases of complete block, by Nembutal (Everett and Sawyer, 1949), have been encountered after administering blocking agents to 4-day cyclic rats at that hour. In 5-day cyclic rats, however, a few observations after injection of Dibenamine even later in the day of proestrus suggested that in many of these animals, stimulation of the hypophysis may be delayed beyond 4 P.M. For example, of two 5-day cyclic females receiving Dibenamine as late as 6:45 P.M. one was partially blocked.

This question was tested more adequately by giving atropine to 9 proestrous 5-day cyclic Vc rats at 4 P.M. Only 3 had fully ovulated before autopsy the following morning. In 3 others partial blocking occurred: in 2 cases ovulation was in progress at autopsy; in the other ovulation was imminent, for all follicles were in late stages of swelling. In the remaining 3 of the 9 rats, complete block had been accomplished, since no histological evidence of preovulatory swelling was found. Thus it is now more definite that in 5-day cyclic proestrous rats the ovulatory stimulation of the hypophysis is less regularly confined within the 2 to 4 P.M. period than is true of 4-day cyclic animals. The bearing of this information on the principal thesis of this paper will be discussed later.

Series III

Comparative effectiveness of progesterone injections at various hours of the day (diestrus day 3)

The experiments of this series are graphically presented in figure 2. It is apparent that the injection of progesterone at any hour from 8 A.M. to 2 P.M. on the third day of diestrus will almost always induce ovulation during the following night. Only 2 failures (6%) were encountered among 31 rats injected at these hours.

The group of 22 animals injected at 3 to 4 P.M. is, therefore, of particular interest, since about half of them failed to respond. This was almost equally true among those having natural 5-day cycles (10 rats) and those which were potentially 4-day cyclic but in which

the experimental cycles had been retarded by progesterone injected on diestrus day 1 (12 rats). Among the former 10 rats only 6 ovulated overnight, while an additional rat had 1 follicle in late swelling. Among the latter 12 animals only 5 ovulated before autopsy the morning after treatment. In 1 of the 5, ovulation was in progress at autopsy, evidence of partial stimulation. There is no significant difference, therefore,

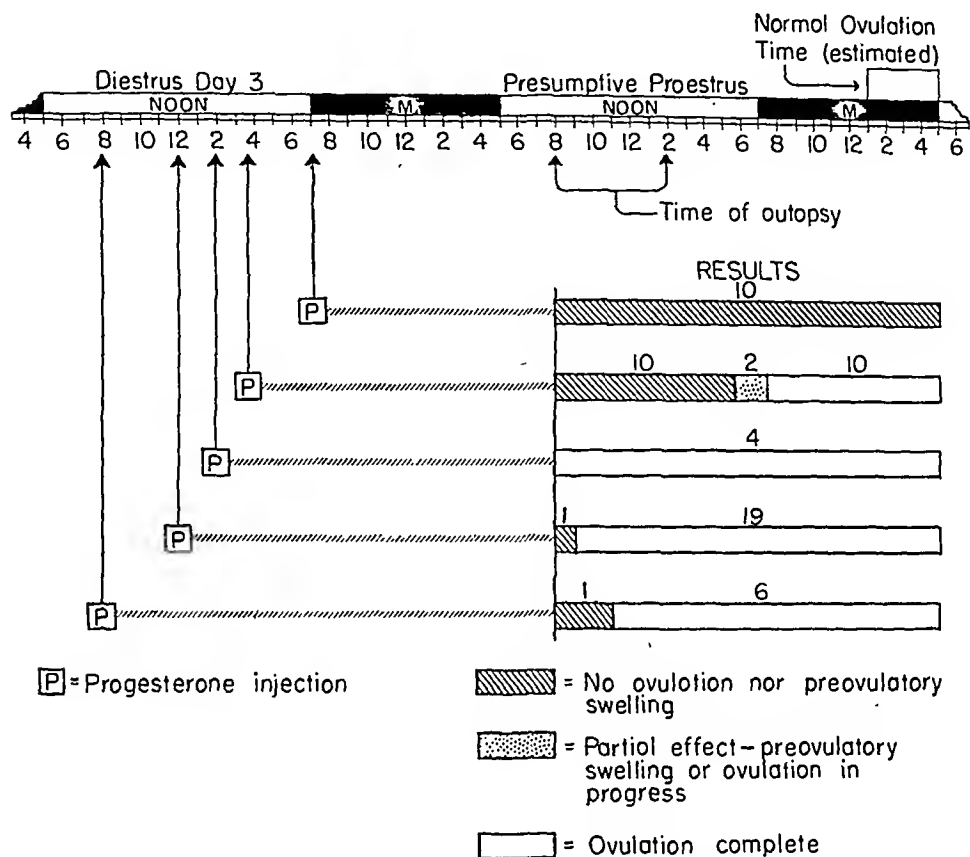


FIG. 2. Graphic representation of the relative effectiveness of progesterone, injected at different hours of the third day of diestrus. Significantly lowered response when delayed until 3-4 P.M. No response when delayed until 6:30 P.M. Numerals over the various segments of the bars under "RESULTS" indicate the respective numbers of rats represented by the proportional lengths of the segments. "Normal ovulation time" indicated above the time scale is estimated from data in Series II.

between natural and artificial 5-day cycles, in the number of animals responding to progesterone after 3 to 4 P.M. The grouping together of data from both varieties of 5-day cycles in the other experimental categories seems entirely justified. (The perfect symmetry of distribution shown for the group as a whole can hardly be taken seriously from a statistical viewpoint, but simply represents our actual observations.)

The remaining group, injected with progesterone at 6:30 to 6:45 P.M., comprises 10 cases among which were 7 five-day cyclic Vc rats

and 3 Va rats with "artificial" 5-day cycles. None ovulated overnight and no histological evidence of preovulatory swelling was found. Since none was autopsied earlier than 10 A.M. we should have found advanced preovulatory swelling, if not complete ovulation, had LH release been effected. This statement is based on the following considerations: (1) Data from Series IV (below) show that progesterone can induce full hypophyseal stimulation within 4 hrs. after injection, under optimal conditions. (2) From the more detailed information in 4-day cyclic rats (Everett, Sawyer and Markee, 1949) we know that ovulation regularly occurs within 11 hours after full stimulation of the hypophysis.

In addition to the 10 definitive cases mentioned in the above paragraph, there were 2 animals which we believe to represent spontaneous advancement of ovulation, not actually resulting from the progesterone treatment. Their new corpora lutea, in histological preparations, are judged to be too far advanced to have resulted from hypophyseal stimulation much later than 4 P.M. Following progesterone treatment at 6:30 P.M. little stimulation could be expected until the 8:30-10:30 P.M. interval (allowing a latent period of about 2 hours after injection; cf. Series IV). Hence, it is highly probable that their hypophyses were spontaneously stimulated before the progesterone could have acted.

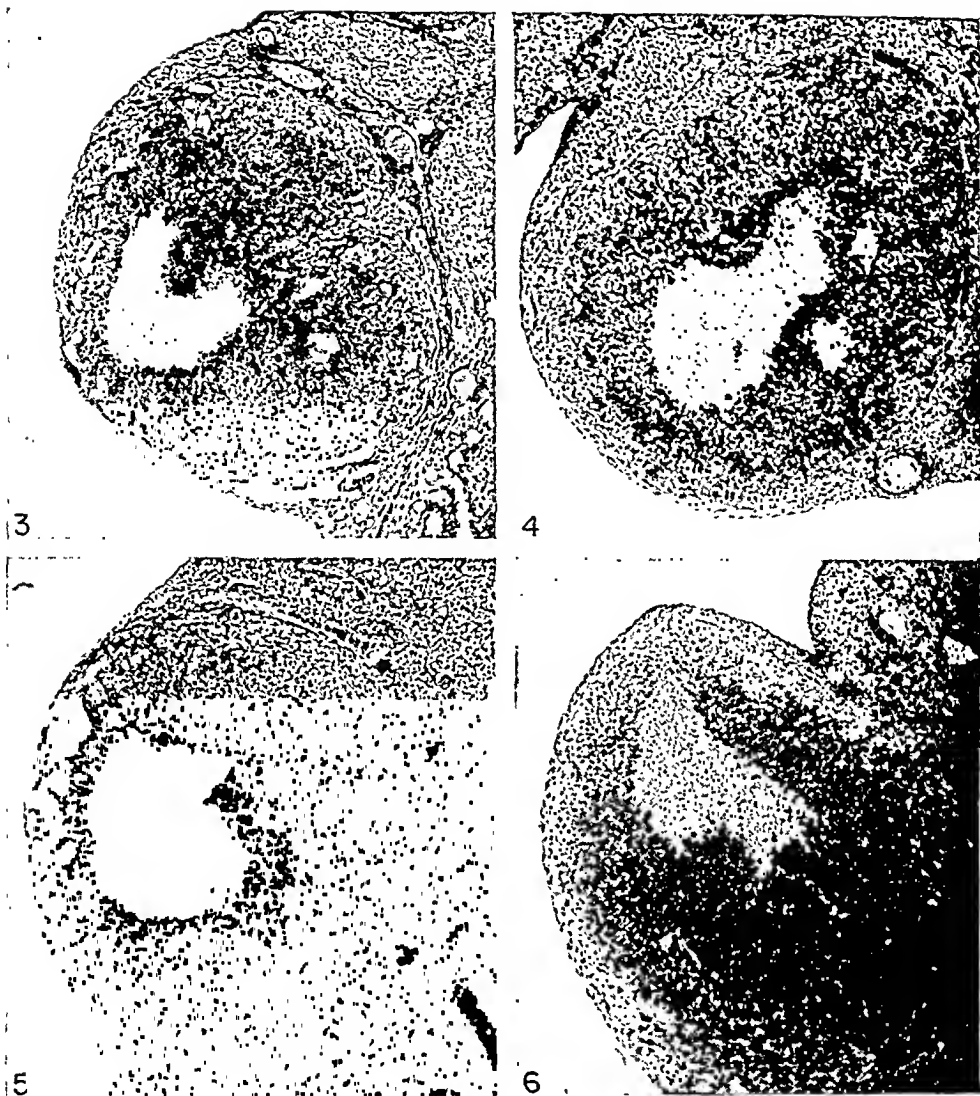
Indications are that at some time late in the afternoon or early evening of the third day of diestrus the 5-day cyclic rat becomes refractory to progesterone. The hour at which refractoriness begins varies considerably among different individuals: in some it may begin as early as 5 P.M.; in others later, but probably not long after 8 P.M.

Series IV

Interval between progesterone injection and response as a function of time of day

A comparison was made between corpora lutea (from Series III) induced by 8 A.M. injection of progesterone and those induced by injection at noon or 2 P.M., taken at similar hours of autopsy (figs. 5 and 6). These were also compared with corpora lutea in 4-day cyclic rats autopsied at comparable hours (figs. 3 and 4). None of the progesterone-induced corpora was more advanced than the latter. This suggested that the early injection of progesterone may not result in proportionately early stimulation of the hypophysis.

The use of atropine as a dependable agent for blocking neurohumoral stimulation of the pituitary gland afforded a means of testing this possibility. By injection of progesterone early or late and by injecting atropine at varying intervals afterward, we found clear evidence that hypophyseal stimulation in response to progesterone is a function of the time of day and that it is not primarily related to the hour of progesterone administration.



FIGS. 3 and 4. New corpora lutea in ovaries of 4-day cyclic rats at 8 A.M. and 10:30 A.M., resp., on the morning after ovulation. 80 \times .

FIG. 5. Progesterone-induced corpus luteum found at 8 A.M. on the morning after ovulation. Progesterone injected exactly 24 hours earlier, on diestrus day 3. Note similarity to fig. 3. 80 \times .

FIG. 6. Progesterone-induced corpus luteum found at 10:30 A.M. on the morning after ovulation. Injection at 2 P.M. on the preceding afternoon, on diestrus day 3. Note similarity to fig. 4. 80 \times .

Nine rats received progesterone at 8 A.M. on diestrus day 3 and were atropinized 6 hrs. later at 2 P.M. (fig. 7). None ovulated overnight. In 2 cases there was evidence of slight LH action, as their ovaries contained, respectively, 1 and 2 follicles in preovulatory swelling. In contrast with these findings are numerous positive results obtained when progesterone was injected at noon or 1 P.M., followed by atropine at only a 4-hour interval. Ten rats received progesterone at 12 noon and atropine at 4 P.M. Four ovulated "normally" overnight and

2 were partially stimulated as evidenced, respectively, by 1 and 5 follicles in preovulatory swelling. Thirteen rats received progesterone at 1 P.M. and atropine at 5 P.M. A still higher proportion of these animals fully ovulated overnight (7/13). In one additional animal ovulation was in progress at the time of autopsy (5 tubal ova, 6 freshly ruptured follicles, 6 follicles in preovulatory swelling), indicating that the hypophysis had been incompletely stimulated before the atropine took effect. Five of the 13 rats were completely blocked.

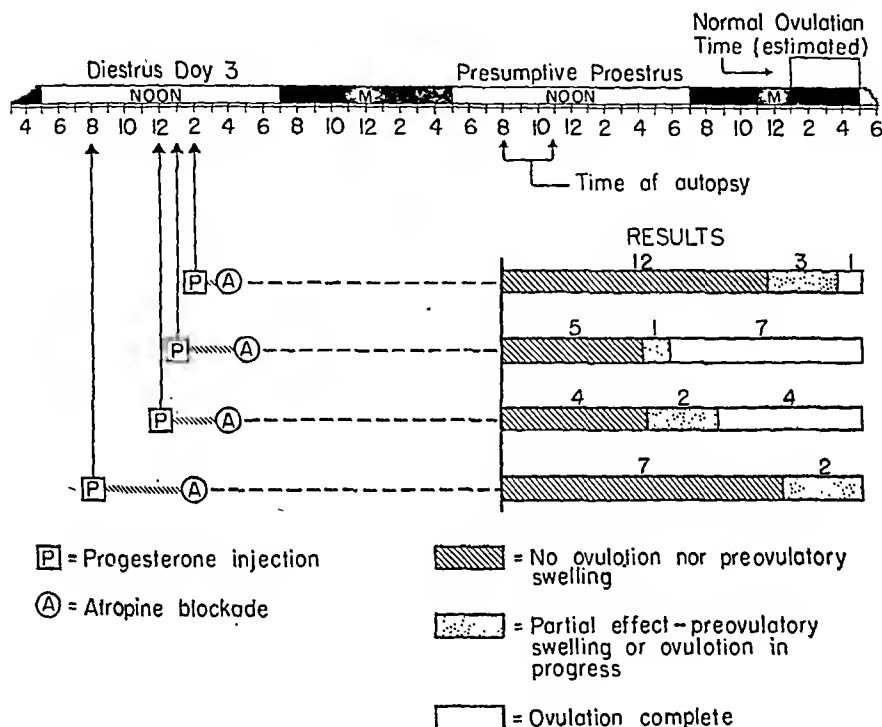


Fig. 7. Graphic representation of results of atropine blockade at various intervals after progesterone injection at different hours of the day (diestrus day 3). Numerals over the segments of the bars under "RESULTS" indicate the respective numbers of rats represented by the proportional lengths of the segments.

In these animals, therefore, no effective stimulus had reached the hypophysis before 5 P.M.

To learn whether progesterone can exert its effect in even less than 4 hours, 16 rats were given progesterone at 2 p.m. (on diestrus day 3 of artificial 5-day cycles) and atropine 2 hours later. On the following morning, 1 of the 16 had ovulated completely and a second rat was in the process of ovulating at the time of autopsy, having 4 tubal ova, 6 freshly ruptured follicles and 3 follicles in preovulatory swelling. Two others each had 1 follicle in preovulatory swelling. The remaining 12 rats gave no evidence of preovulatory stimulation. Their ovaries

contained full sets of large, growing follicles of which the ovocyte nuclei were in the "resting" state. Four cases of pituitary activation among 16 trials are not very significant since there is a possibility of occasional spontaneous advancement of the cycle (cf. Materials and Methods). Allowing a 10% probability for that event, the likelihood is considerable ($p > 0.06$) that it might occur in at least 4/16 cases. We therefore judge that a latent period of over 2 hours follows the subcutaneous injection of progesterone in oil. If the rat is sensitive during the second 2-hour period, pituitary activation will probably be completed during that interval.

Considering all of these progesterone-atropine experiments together, it is evident that regardless of how early in the day progesterone is injected, the neurohumoral response does not begin until about 2 P.M. or later. In only about half of the animals is hypophyseal stimulation largely complete before 4 to 5 P.M. In the others little, if any, stimulation has occurred at such time. This distribution of individual periods of stimulation before and after 4 to 5 P.M. is not unlike that found in Series II (above) for 5-day cyclic rats in normal proestrus. Both distributions, however, are dissimilar to that seen in 4-day cyclic rats in proestrus, where 25 of 27 animals have shown at least partial stimulation before 4 P.M.

DISCUSSION

There is nothing surprising in the finding that a neural mechanism is involved in LH release in response to progesterone administration. The conclusion had earlier been reached (Everett, 1948) that progesterone and estrogen act synergistically in the induction of LH discharge in rats. Sawyer, Everett and Markee (1949) had demonstrated that ovulatory activation of the hypophysis in response to estrogen (in pregnant rats) can be blocked by either Dibenamine or atropine. It seemed highly probable, therefore, that these blocking agents would similarly prevent the advancement of ovulation by progesterone.

Of considerably more importance is the disclosure of a 24-hour rhythm of sensitivity in the neural LH-release apparatus. In Series III and IV we found that on the third day of diestrus in 5-day cycles, sensitivity to progesterone is limited to a period of a few hours. This period is approximately 24 hours earlier than the period of "spontaneous" stimulation of the hypophysis during proestrus. The response to progesterone is related largely to time of day and not primarily to the hour of progesterone injection. If the hormone happens to be present in adequate amount during the period of sensitivity on diestrus day 3, stimulation of the anterior lobe takes place. Otherwise another day will elapse before stimulation occurs.

Confirmatory evidence of quite different nature, also disclosing such a 24-hour rhythm in some element of the neural LH-release apparatus, has already been presented in a preliminary paper (Everett and Sawyer, 1949). Brief Nembutal sedation of 4-day cyclic rats

during the critical hours of sensitivity on the day of proestrus will regularly delay LH release for a full 24 hours. Repeated injection of Nembutal during critical hours on the second day will then delay release another 24 hours. The mature follicles persist and estrogen secretion continues meanwhile, as evidenced by the vaginal smears. There must be, then, a continuing steady secretion of gonadotrophin (FSH + LH) at moderate levels. The one deficiency is a stimulus from the hypothalamus sufficient to discharge adequate amounts of LH (or total gonadotrophin) to cause preovulatory swelling and ovulation. In the present experiments we are dealing essentially with this same mechanism. When the cycles are dated from the first day of diestrus, the day of proestrus in the 4-day cycle compares with the third day of diestrus in the 5-day cycle. On this day the hypophysis of a 5-day cyclic rat is fully capable of releasing an ovulating quantity of luteinizing hormone, but the neural stimulus for release is normally one day "late" in arriving.

There is at hand some evidence to indicate the anatomical localization of an hypothalamic center which is specifically essential for the cyclic ovulatory discharge of LH. Dey *et al.* (1940-1943) placed lesions in various parts of the hypothalamus in female guinea pigs. Certain bilateral lesions, restricted to the rostral hypothalamus, resulted uniformly in constant estrus, with persistent follicles as a consistent feature of ovarian histology. Intact tuber cinereum and stalk were essential to the production of constant estrus, as their destruction led always to anestrus. Hillarp (1949) has recently shown in female rats that certain bilaterally symmetrical lesions in the rostral hypothalamus similarly result in constant estrus. Equally effective are: (1) relatively diffuse lesions in the anterior hypothalamic area immediately rostral to the paraventricular nuclei, or (2) more discrete lesions placed caudally between the paraventricular nuclei and median eminence. The ovaries of such animals are reported to contain many follicles of various sizes and an extensive fat-laden interstitium. Gonadotrophin secretion is apparently steady, but at a subovulatory level. The deficiency lies in the absence of adequate stimulation for full, ovulatory secretion of "LH." It is probably true, therefore, that the cyclic element of the LH-release apparatus resides in, or operates through, the anterior hypothalamic area. The present results show that one of its functional attributes (in the rat, at least) is an intrinsic 24-hour rhythm. A number of other functional characteristics were previously discussed by Everett, Sawyer and Markce (1949), where we spoke more abstractly of a rhythmic hypothalamic center whose activity leads to discharge of an "ovulation stimulus" to the hypophysis. We are now inclined to regard the center in the rostral hypothalamus as the origin of this spontaneous stimulus (Everett, 1950). That part of the mechanism represented by the tuber cinereum, median eminence and hypophyseal portal circulation, operates as a final pathway to the pituitary gland, according to this view.

The specific site or sites of action of progesterone and estrogen in this system cannot as yet be defined. Both substances have central neural effects, e.g., incitement of estrous behavior in which a synergism of the two steroids is well recognized (Beach, 1948). In the present study we have seen that injection of progesterone on the first day of diestrus in 4-day cyclic rats not only retards the cycle one day, but often modifies the critical hours during which pituitary activation can occur on the second day following. Without the earlier retarding dose of progesterone, spontaneous activation would usually occur on that day (proestrus) between 2 and 4 P.M. In the retarded cycle, however, we find that on that same day (now the third day of diestrus) the LH-release apparatus of about half of the animals is insensitive to progesterone until after 4 P.M. This change is quite evidently an effect of progesterone on the central nervous system, and probably on the anterior hypothalamic center. By analogy, this shift of the diurnal rhythm resembles a resetting to a new hour of the alarm control of a 24-hour clock.

With respect to possible mechanisms whereby progesterone (or estrogen) may act to induce ovulation, we entertain three alternative hypotheses. The first of these takes the extreme view that neurohumoral stimulation of the hypophysis occurs at a regular time each day and that the response of the gland depends entirely on local modification of its threshold of activation by the sex steroids. The principal evidence favoring this interpretation is the recent observation in the rabbit (Sawyer and Markee, 1950) that copper acetate instilled directly into the anterior lobe tends to induce LH release and that this effect is facilitated by estrogen. The principal evidence suggesting that progesterone, at least, acts at the neural level and not only in the gland, is the fact that this agent will induce ovulation in rats in which a state of persistent estrus has been induced by continuous illumination (Everett, 1940, and unpublished). At present the simplest explanation of such "light estrus" is that continuous illumination inhibits the operation of the cyclic element in the hypothalamic apparatus, either by destroying the diurnal rhythm or by preventing its influence on the final pathway. If such is true, then the action of progesterone administered during continuous light must be at the neural level, at least in part.

The second hypothesis considers that a principal site of action of the sex steroids is in the tuberal region and (or) the median eminence, where they serve to lower thresholds to a variety of afferent impulses. We may then assume for the moment that given adequate steroid levels at these sites, stimuli from the 24-hour "clock" administer the *coup de grâce*. This hypothesis resembles the first in that it assumes excitatory activity proceeding from the rostral hypothalamus regularly each day.

The third hypothesis assumes that the center in the rostral hypothalamus, is characterized by an (intrinsic?) diurnal rhythm of

sensitivity, but that its own threshold of excitation is determined by local action of sex steroids. In the 5-day cyclic rat, it may then discharge excitatory impulses to the tuber cinereum-median eminence apparatus on the third day of diestrus if progesterone is introduced well before the critical hours of sensitivity. Lacking such treatment, as when progesterone is injected too late in the day, or not at all, the discharge does not occur until another day has passed. This hypothesis does not deny the additional action of estrogen and progesterone at the sites mentioned in the other hypotheses but simply regards the steroids as being critical for determining the actual exciting discharge from the rostral hypothalamus.

There is at present little choice between the second and third hypotheses. The latter, perhaps, would allow more ready comparison of spontaneously ovulating species with those which require the copulatory reflex. The median eminence and portal vascular connection to the pars distalis constitute a final pathway which is apparently common to both rat and rabbit (cf. Harris, 1948). The rabbit lacks the rhythmic element of the rostral hypothalamus, one may say. Activation of the final pathway in such species depends on afferent impulses normally associated with coitus. In the rat the rostral center discharges spontaneously to the final pathway every 4 or 5 days, substituting for, or more correctly, obscuring the reflex mechanism in so far as ovulation is concerned. Reflex coital stimulation leading to ovulation does exist in rats, but is demonstrable only under special conditions: during spontaneous persistent estrus (Everett, 1939, and later unpublished data) and during persistent estrus induced by continuous light (Dempsey and Searles, 1943; Everett, unpublished). It will be of considerable interest to learn whether Hillarp's (1949) constant-estrous preparations retain the reflex mechanism in the absence of the spontaneous stimuli from the rostral hypothalamus. If not, one would be forced to conclude that even the reflex mechanism requires the integrity of that region. It should be recalled that Haterius and Derbyshire (1937) induced ovulation in rabbits by means of weak, localized stimuli from bipolar electrodes placed in the preoptic region.

Putting aside these neurological questions, there are certain more general matters respecting the relationship of progesterone to ovulation which should be discussed. We are led to inquire what may be its general importance in facilitating ovulation in other species than the rat and hen. It seems hardly a coincidence that such closely similar phenomena have been observed in such widely unrelated species. Our present evidence re-emphasizes the critical importance of timing in the rat. In the first place, as was earlier shown by one of us (Everett, 1948), the difference of one day in administration of progesterone can determine whether ovulation is to be retarded or advanced. Now it is apparent that there are only a few hours on the third day of diestrus during which the LH-release apparatus is sensi-

tive to the hormone. Is it not probable that when the factor of timing is better understood, similar activity of progesterone will be disclosed in other species?

There is still much to be learned about the importance of progesterone in such functions even in the rat. Lest it be construed that we believe that the hormone is always essential, we must reiterate evidence to the contrary which has previously been cited (Everett, 1944, b). The first ovulation at puberty necessarily occurs in the absence of luteal tissue. In adult persistent-estrous rats, spontaneous ovulatory cycles sometimes occur long after all recognizable luteal tissue has disappeared. Of course, one must admit that progesterone of extra-luteal origin is not excluded in these cases. Marvin (1947, 1948) was able to induce ovulatory cycles in persistent-estrous rats by daily injection of small amounts of testosterone propionate or of desoxycorticosterone acetate. Hooker and Forbes (1949), however, suggest that these two substances may be partly transformed to progesterone *in vivo*. Unpublished data by one of us (J. W. E.) show that single injections of testosterone propionate (0.05 to 1.0 mg. in oil) on the third day of diestrus will not advance ovulation in 5-day cyclic rats. Neither will desoxycorticosterone acetate unless the dose is increased to 3 mg. Perhaps these acute experiments allowed insufficient time for conversion, in contrast with the chronic experiments of Marvin.

One may inquire what evidence there is that progesterone is actually produced in rats at a sufficient length of time prior to ovulation to influence LH discharge. Astwood (1941) postulated on experimental grounds that progesterone is secreted by the ovarian follicles during preovulatory swelling. However, swelling does not begin until several hours after the discharge of LH; we are concerned here with events preceding that discharge. Effective amounts could probably not be secreted by the growing follicles before swelling, at least not under the stimulus of luteotrophin (lactogen; cf. Everett, 1944, b). The more plausible source is the luteal tissue of the preceding cycle, in which cytochemical evidence of secretory activity is apparent early in the day of proestrus (Everett, 1945) in normal rats. No comparable evidence of luteal secretion was found in certain rats which were especially subject to persistent estrus, either spontaneous or light-induced. The latter observation is of major significance in support of a protective rôle for progesterone.

Whether or not it is always essential to LH release, progesterone certainly favors this process and tends to counteract inhibitory influences such as continuous illumination (Everett, 1940). In this protective rôle it apparently acts at the hypothalamic level facilitating the normal functioning of a diurnally sensitive mechanism.

SUMMARY

Earlier studies demonstrated that progesterone, administered to 5-day cyclic rats on the third day of diestrus, will advance ovulation

about 24 hours. It is now shown that this effect is subject to blockade by either Dibenamine or atropine, indicating that the effect is mediated by a neurohumoral mechanism.

The relative effectiveness of progesterone injection at different hours of the day was examined. The results show that sometime in the late afternoon or early evening the animals become refractory to progesterone. This hour varies considerably among different individuals. The variation is of comparable degree in natural 5-day cycles and in "artificial" 5-day cycles (4-day cycles retarded one day by progesterone administered on diestrus day 1).

By injection of progesterone at various hours, followed at different intervals by atropine blockade, it was found that regardless of how early progesterone is administered, neurohumoral activation of the hypophysis does not begin until 2 P.M. or later, becoming complete in about half of the animals before 4-5 P.M. In the others little, if any, stimulation occurs until after that time. The response is primarily related to the time of day and not to the hour of injection.

After subcutaneous injection of progesterone in oil a latent period of at least 2 hours is characteristic. If the animal is sensitive during the second 2-hour period post-injection, full stimulation of the hypophysis may be accomplished during that interval.

The period of sensitivity to progesterone on the third day of diestrus is almost exactly 24 hours earlier than the period of "spontaneous" activation which ordinarily occurs on the next following day (proestrus). This is evidence of a 24-hour rhythm in some element of the LH-release apparatus.

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A PROCEDURE FOR THE STUDY OF FACTORS WHICH AFFECT THE NITROGEN METABOLISM OF ISOLATED TISSUES: HORMONAL INFLUENCES¹

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THE classical approach to the study of the nitrogen metabolism of tissues has been through observations of urinary nitrogen changes. Efforts at more direct measurement have led to studies of NPN fractions in the blood of normal (Harrison and Long, 1940), nephrectomized (Bondy and Engel, 1947), and eviscerated (Frame and Russell, 1946) animals under a variety of conditions. New approaches which have recently been introduced are the analyses of whole tissues before and after experimental manipulation of the animal (White and Dougherty, 1947) and the use of isotopes to calculate breakdown and synthesis rates (Sprinson and Rittenberg, 1949).

Direct in vitro observation of the nitrogen metabolism of tissues has been discouraged by the finding that during incubation there is always a net loss of nitrogen from the tissue slices. However, if the loss of nitrogen during incubation is a constant, reproducible quantity, then the influence of various factors upon the metabolism of tissues may be studied using the values found under standardized conditions as a reference point. Moreover, it has recently been claimed that labeled nitrogen is incorporated into protein in vitro (Frantz, Loftfield and Miller, 1947), suggesting that although the breakdown of protein predominates over synthesis under in vitro conditions, comparison of the rate of loss of nitrogen from tissues under various conditions may supply useful data concerning factors which are of importance in the nitrogen metabolism of the tissues.

Bernheim and Bernheim (1946) have introduced evidence which supports the assumption that measurement of the increase of nitrogen in a bicarbonate buffer in which tissues are incubated is a reflection of metabolic processes which are taking place in the surviving slices. In this paper are reported the results of a more intensive study of this system. Precautions which must be taken in order to obtain consistently reproducible measurements of the rate of liberation of nitrogen

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lactate to their drinking water. Only those animals which showed marked retardation of growth were used. The thyroidectomized rats which received thyroxine were given a single subcutaneous injection (20μ gm.) 18 hours before the experiment and received no food thereafter.

RESULTS

Removal of tissue fragments. The necessity of removing fragments which separated from the suspended liver slices during incubation is shown by the data in Table 1. The nitrogen lost from the slices was calculated by subtraction of the nitrogen content of the slices at the time intervals shown from the nitrogen content of fresh slices. The

TABLE 1. MG. OF TOTAL NITROGEN TRANSFERRED TO KREBS-RINGER PHOSPHATE BUFFER FROM 100 MG. (WET WEIGHT) OF LIVER SLICES

	n	15 min.	195 min.	ΔN
N lost from slices	8	$0.97 \pm .06$	$1.44 \pm .11$	$0.47 \pm .09$
N gained by medium	8	$0.89 \pm .05$	$1.22 \pm .11$	$0.33 \pm .10$
N gained by medium- fragments removed	4	$0.84 \pm .05$	$1.00 \pm .04$	$0.16 \pm .03$
N* gained by medium- fragments removed	11	$0.84 \pm .02$	$1.18 \pm .02$	$0.34 \pm .03$

* Bicarbonate buffer.

nitrogen gained by the medium was obtained directly by analysis of aliquots of the medium since no nitrogen was present at the start of the experiment. It will be noted that fragments which separated from the slices during incubation prevented the obtaining of true aliquots from the uncentrifuged medium since the nitrogen lost from the slices was invariably greater than that obtained by analysis of the medium. That no nitrogen was lost from the system during the course of the experiments was proved by comparison of the nitrogen content of control slices with nitrogen recoverable upon analysis of the entire contents of flasks after 3 hours of incubation. In six such experiments, the average recovery was 100.4 per cent (range 98.6-101.9) of the values obtained from duplicate analyses of control slices.

Comparison of the nitrogen added to the medium during the incubation period as shown in the last column of Table 1 with and without centrifugation indicates that fragments from the liver slices contributed at least 50 per cent of the nitrogen measured during the interval from 15 to 195 minutes. Fragmentation was exceedingly variable as is shown by the large standard error in 8 experiments in which the fragments had not been removed as compared with the small standard error in 4 experiments in which the medium was centrifuged prior to nitrogen analysis.

USE OF 15 MINUTE EQUILIBRATION PERIOD

A considerable quantity of nitrogen entered the medium during the first 15 minutes of incubation (see Table 1). Studies at 5 minute

intervals indicated that this rapid phase of nitrogen liberation ceased after 15 minutes. Considerable data have been accumulated, all of which indicate that the nitrogen lost from the tissues during this time is not of biological significance. For example, alteration of the osmotic pressure, ionic strength or the temperature of the system failed to affect the 15 minute value. Fasting the animals for 3 days prior to removal of the liver also failed to influence the results. Positive evidence that the nitrogen transferred from the slices to the medium during the first 15 minutes of aerobic incubation resulted almost entirely as a consequence of the slicing process is afforded by the following experiments: (a) In two experiments, when fresh slices were incubated for 15 minutes in a medium in which a set of slices had

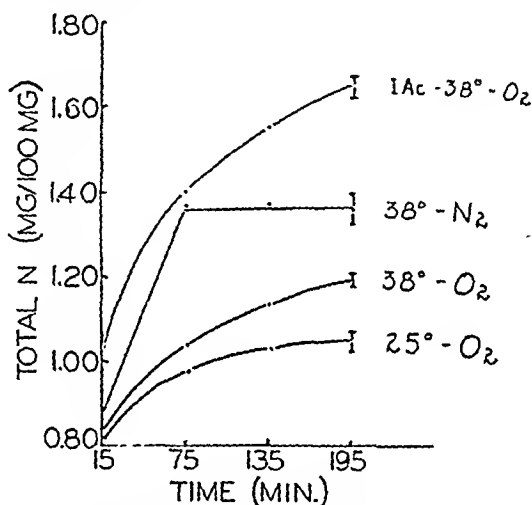


FIG. 1. Release of total nitrogen from liver slices into Krebs-Ringer bicarbonate buffer.

previously been equilibrated and then removed, there was an increment of total nitrogen in the medium from the fresh slices exactly equal to that obtained from the first set of slices. (b) On the other hand, when slices were transferred after 15 minutes to fresh media and re-equilibrated, the addition of nitrogen to the medium during the second 15 minute period was slight. (c) The 15 minute total nitrogen value in the case of diaphragm increased as the total number of pieces into which the diaphragm was cut was increased.

The advantages of using the 15 minute point as a zero value are four-fold: (a) the effects of differences in slicing are minimized, (b) the effect of varying quantities of residual blood in the tissues is eliminated, (c) the addition of nitrogen-containing substances such as hormone preparations is internally controlled and (d) the true significance of small changes which might otherwise not be detected is revealed by elimination of nitrogen which has entered the medium as a result of non-metabolic processes.

REPRODUCIBILITY OF VALUES

Included in Table 1 are data obtained from liver slices of normal animals incubated in bicarbonate medium, to show the reproducibility of the values observed. It will be noted that only 4 or 5 animals are needed to establish the variability of an experimental series, *i.e.*, the standard error approaches its minimum value rapidly.

EVIDENCE THAT NITROGEN LIBERATED FROM TISSUES
REFLECTS METABOLIC PROCESSES

Three series of experiments were performed to test whether the increase of nitrogen in the medium after a 15 minute equilibration

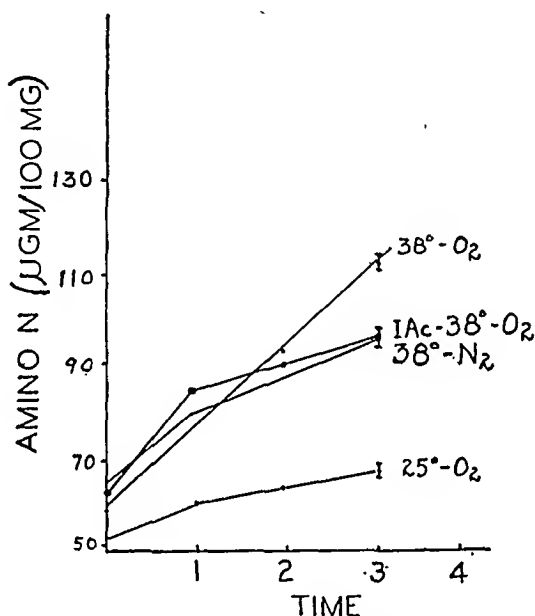


FIG. 2. Appearance in Krebs-Ringer bicarbonate buffer of amino nitrogen from liver slices.

period was influenced by factors known to affect the metabolism of tissues. Liver slices were incubated (a) in the absence of O₂, (b) at reduced temperatures (15–25°C) and (c) in the presence of iodoacetate. The results for total N are shown in Figure 1 and those for amino N in Figure 2. The increase of total N in the medium during the experiments, when compared with the values in a series carried out under normal conditions, was increased significantly ($P < .01$) by anaerobic incubation and by the use of iodoacetate and was decreased at reduced temperatures. There was an inhibition of amino N production under each of these conditions.

EFFECTS OF HORMONES

The effects of adrenalectomy, thyroidectomy and hypophysectomy upon the liberation of total N from diaphragm and liver slices in the

interval between 15 and 195 minutes, are represented as open bars in Figure 3. In the case of diaphragm, the results for each series of operated animals were significantly different from those of the control series ($P < .01$). For liver, no difference was found between the normal series and the adrenalectomized animals whereas the hypophysectomized and thyroidectomized animals had a marked diminution in the liberation of total N into the medium during the 3 hours of observation.

The effects of the injection of thyroxine into hypophysectomized and thyroidectomized rats and of adrenal cortical extract into ad-

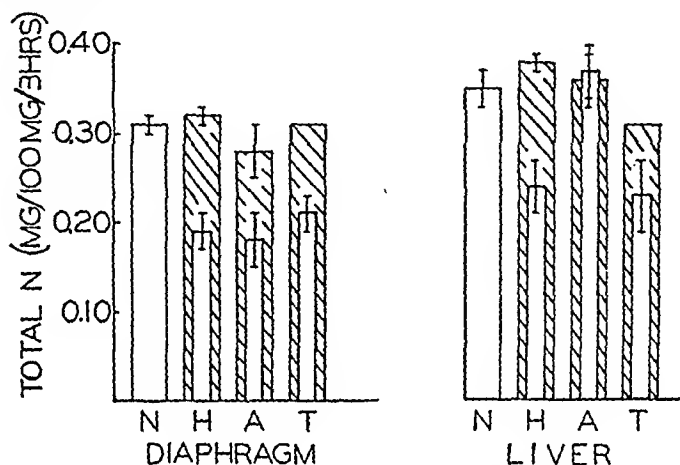


FIG. 3. Release of total nitrogen from diaphragm and liver slices during interval from 15 to 195 minutes. N—normal. H—hypophysectomized, A—adrenalectomized, T—thyroidectomized. Open bars represent untreated animals, shaded areas show effect of hormone treatment upon operated animals.

renalectomized rats are demonstrated for diaphragm and liver in the shaded bars superimposed upon each corresponding untreated, operated series (Figure 3). The treated animals showed a return to or near the level of nitrogen output observed in the unoperated series in each instance. The liberation of nitrogen from the liver of adrenalectomized rats, which was identical with that of the unoperated series, was unaffected by adrenal cortical administration.

Similar effects upon the formation of amino N may be seen in Figure 4. The open bars represent the appearance of amino N during a 3 hour incubation period (15 to 195 minutes) in normal, and untreated, operated animals, and the superimposed shaded bars demonstrate the effect of the administration of the appropriate hormone in each instance. As with total N, the amino N values for each series differ significantly ($P < .01$) from that of the unoperated series except that, once again, adrenalectomy failed to influence the response of liver slices. The injection of thyroxine into hypophysectomized and thyroidectomized rats increased the formation of amino N to

the unoperated level. Aqueous adrenal cortical extract did not completely restore the amino N production from the diaphragm of adrenalectomized rats to normal levels, possibly because of the use of an aqueous rather than the more potent lipo-adrenal preparations and because arbitrary selection of a time interval may not have permitted observation of the maximal effect, and had no influence upon the liberation of amino N from liver slices.

An earlier series of experiments upon adrenalectomized rats (not paired with rats treated with cortical extract) which were performed some time before the series shown in Figures 3 and 4, yielded values

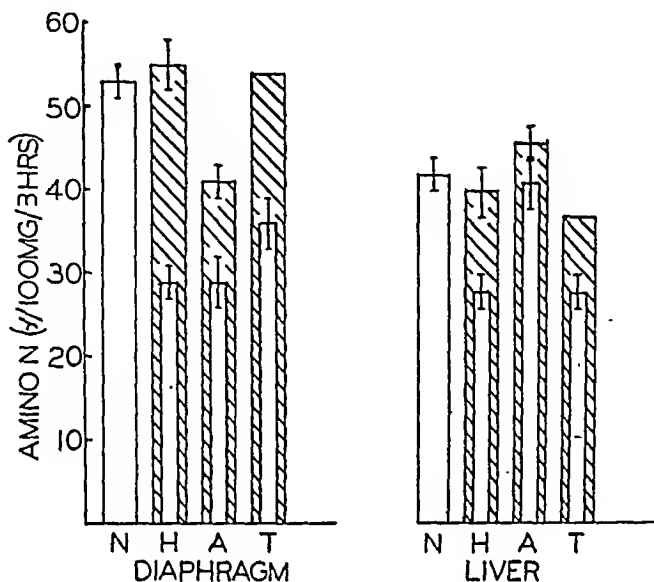


FIG. 4. Appearance in Krebs-Ringer bicarbonate buffer of amino nitrogen from diaphragm and liver slices during interval from 15 to 195 minutes. Abbreviations as in Figure 3. Approximately the entire original amino N content of the diaphragm and liver was recovered from the medium during the 15 minute equilibration period. The values shown above, therefore, represent newly formed amino acids.

for the liberation of total and amino N from the diaphragm somewhat higher than those reported in this communication. The cause of this discrepancy is now being investigated. However, the difference in the two series was only quantitative, the values in each instance being significantly different from that of the control, unoperated series.

The net increment of amino N in the medium upon incubation of diaphragm from normal animals was significantly greater than that of liver slices ($P < .01$). The formation of urea by liver slices may possibly explain this apparently greater proteolytic power of diaphragm.

Control total N values of fresh tissues obtained from adrenalectomized, hypophysectomized and thyroidectomized animals were almost identical with the amount of nitrogen found in fresh tissue from unoperated rats both on a wet and a dry weight basis per 100 mg. of

tissue. The differences in the liberation of nitrogen from operated animals as compared to the normal series, therefore, cannot be explained by variation in the initial nitrogen content of the tissues.

DISCUSSION

In order to carry out upon isolated tissues quantitative nitrogen balance studies that are biologically meaningful, it is necessary that the values obtained are reproducible, and the phenomena must be such that diffusion and the attaining of nitrogen equilibrium between tissue and medium do not suffice to explain the results observed. The data presented clearly demonstrate that the liberation of nitrogen from liver slices and diaphragm and the formation of amino acids by these tissues are highly reproducible phenomena which are well suited to quantitative study. The evidence also strongly supports the assumption that the measurements reflect metabolic activity of the tissues and are not the result of purely physicochemical processes since the quantity of total and amino N which accumulates in the medium is altered significantly, as compared to aerobic incubation at 38° C, when the conditions of incubation are changed in a manner known to affect the metabolism of tissues (i.e., the absence of O₂, low temperature, addition of iodoacetate). In addition, the results obtained from hypophysectomized, thyroidectomized and adrenalectomized animals as well as the effect of hormone therapy confirm the metabolic dependency of the processes that are being measured.

A state of "relative" positive nitrogen balance, as compared to the normal series, was found in liver slices and diaphragm from adrenalectomized, hypophysectomized and thyroidectomized animals, except that adrenalectomy had no effect upon the liver values. The administration of adrenal cortical extract to the adrenalectomized rats and of thyroxine to hypophysectomized and thyroidectomized animals induced an increased loss of total N and the formation of more amino N as compared to operated, untreated controls. These observations are in accord with *in vivo* findings concerning the manner in which these hormones affect the nitrogen balance of the whole animal. The amino N data are likewise in accord with *in vivo* findings in eviscerated rats (Bondy, 1949).

The procedure as described, on the basis of the experiments reported above, appears to be well suited to the study in isolated tissues of the influence of hormones, substrates, inhibitors and many other factors which may be of interest in nitrogen metabolism.

SUMMARY

A procedure has been devised for the quantitative study of the transfer of nitrogen from surviving liver slices and rat diaphragm to an artificial medium during incubation. Under the conditions described the results obtained are highly reproducible.

Evidence is presented that the rate of release of nitrogen from the tissues is a reflection of the metabolic activity of the surviving slices.

A marked diminution was found in the release of total N from and the production of amino N by, the diaphragm of adrenalectomized, thyroidectomized, and hypophysectomized rats as compared to unoperated animals. Liver slices from hypophysectomized and thyroidectomized rats also liberated less total N and formed less amino N than did normal liver slices whereas liver slices from adrenalectomized rats were unaffected.

The administration of thyroxine to thyroidectomized and hypophysectomized rats and of adrenal cortical extract to adrenalectomized animals restored total N release and amino acid production to or toward the values obtained in unoperated animals.

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THE EFFECT OF THE ADRENAL AND THYROID GLANDS UPON THE RISE OF PLASMA AMINO ACIDS IN THE EVISCERATED RAT

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THE mechanism of action of the adrenal cortical hormones upon the nitrogen metabolism has not as yet been elucidated. Previous observations (Engel, Schiller and Pentz, 1949; Bondy, Engel and Farrar, 1949) have suggested that their chief action is probably to promote the breakdown of protein to amino acids, rather than to alter the rate of deamination of amino acids and the formation of urea. Since urea is formed chiefly in the liver, it seemed possible that further information bearing on this point might be obtained by studying the effects of the adrenocortical hormones upon the nitrogen metabolism of the liverless animal.

It has also been shown (Rupp, Paschkis and Cantarow, 1949) that the thyroid exerts a protein-catabolic effect under certain circumstances. Previous workers have suggested that this effect may be separate from the effect of the adrenal cortex (White and Dougherty, 1947). An attempt was made to verify this contention in the present experiments.

METHODS

Adult male Sprague-Dawley rats weighing from 300 to 350 gms were used in all experiments. Adrenalectomy was performed by the lumbar route in one stage. Desoxycorticosterone acetate pellets were implanted subcutaneously at the time of operation. All adrenalectomized animals were eviscerated within 5 to 8 days after adrenalectomy. The adrenal area was examined after death, and animals with accessory or residual adrenal tissue were discarded. Thyroidectomy was performed in the usual fashion under ether anesthesia. Thyroidectomized rats were offered calcium chloride-calcium lactate mixture in their drinking fountains. Thyroxin² was administered daily subcutaneously. Adrenocortical extract³ was given subcutaneously in three hourly doses, starting at the time of evisceration. All animals were given glucose in saline subcutaneously as needed to maintain a normal

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³ Wilson's aqueous adrenal cortex extract.

blood glucose level. Large doses of glucose were given to two groups, by the administration of 0.5 gm. of glucose as 50% solution in saline into the vena cava at the end of the evisceration procedure.

Eviscerations were carried out by the technic of Russel, (1942), which removes the entire intestinal tract from esophagus to rectum, including the spleen, and ties off the hepatic artery and portal vein, thus isolating the liver from the circulation, but leaving the organ in the body. Previous studies have shown that the liver, thus isolated, is removed from the body economy. The animals were kept in a constant temperature box after operation, and their body temperature maintained at $100 \pm 1^\circ$ F. The animals were bled from the tail one and four hours after evisceration and the oxalated blood centrifuged. Plasma amino acid nitrogen determinations were performed by the method of Frame, Russell and Wilhelmi, (1943), and Russell (1944).

RESULTS

The results are shown in Table 1. Adrenalectomy caused a reduction in the rate of rise of plasma amino acid nitrogen. This reduction

TABLE 1. EFFECTS OF VARIOUS ENDOCRINE MANIPULATIONS ON THE RATE OF RISE OF AMINO ACID NITROGEN IN THE PLASMA OF THE EVISCERATED RAT

Change of Concentration, mg./100 ml., from the first to the fourth hour after evisceration

Treatment	Number	Rise of amino nitrogen, mg./100 ml.	p compared to untreated controls
1. None	11	$16.0 \pm .74$ †	
2. 0.1 ml. ACE*/100 gm. in 3 hourly doses	8	$16.5 \pm .86$	> .50
3. 0.5 ml. ACE*/100 gm. in 3 hourly doses	6	$21.9 \pm .76$	< .01
4. Adrenalectomy	5	$9.1 \pm .59$	< .01
5. Adrenalectomy + 0.1 ml. ACE*/100 gm. in 3 hourly doses	5	15.3 ± 1.03	> .50
6. 0.5 gm. glucose intravenously	3	17.8 ± 1.48	> .05
7. 0.5 gm. glucose intravenously + 0.5 ml. ACE*/100 gm. in 3 hourly doses	5	14.5 ± 1.00	> .05
8. Thyroidectomy	6	$12.2 \pm .85$	< .02
9. Thyroidectomy + 0.1 ml. ACE*/100 gm. in 3 hourly doses	7	$13.0 \pm .84$	< .02
10. Thyroidectomy + 20 µg. thyroxin/day for 7 days	8	$15.6 \pm .75$	> .50
4 vs 5, $p = < .01$			
8 vs 10, $p = < .02$			
3 vs 7, $p = < .01$			

* Wilson's aqueous adrenal cortical extract.

† mean \pm standard error of the mean.

could be returned to normal by the administration of 0.1 ml. of adrenocortical extract per 100 gm. body weight per hour for three doses. In the intact animal, this dosage of adrenocortical extract failed to alter the rate of change of the amino acid nitrogen. A five-fold increase the dose, however, produced a significant increase in the rate.

Since it has been shown by Engel, Schiller and Pentz (1949) that the increase of urea formation following adrenocortical extract can be abolished by the administration of glucose, the effects of this pro-

cedure were investigated in the present study. It was found that the increased accumulation of plasma amino acid nitrogen resulting from 0.5 ml. of adrenocortical extract per 100 gm. per hour could be completely abolished by the administration of 0.5 gm. of glucose intravenously at the time of evisceration.

Thyroidectomy also reduced the rate of accumulation of plasma amino acid nitrogen. This effect might have been due to the reduced activity of the adrenal cortex resulting from the general reduction of metabolism of all tissues following thyroidectomy. In order to test this possibility, thyroidectomized animals were given 0.1 ml. of adrenocortical extract per 100 gm. per hour, a dose sufficient to correct the effects of complete adrenalectomy. This dosage of adrenal cortical substances failed to correct the deficit present in the thyroidectomized animals. Thyroxin, however, in dosage of 20 ug per day for 7 days, completely corrected the deficit.

DISCUSSION

The experiments with adrenalectomized animals, and animals given adrenocortical extract indicate that alterations of the nitrogen metabolism can be produced by the adrenal cortex in the absence of the liver. These observations are in agreement with those of Ingle, Prestrud, and Nezamis, (1948) upon adrenalectomized eviscerated rats. Since urea formation is essentially absent in the liverless animal, these effects may be divorced entirely from deamination and urea formation. Protein breakdown and resynthesis, however, continue to occur in the absence of the liver and gastrointestinal tract. The present observations, therefore, offer additional support for the concept that the action of adrenocortical hormones is concerned with protein breakdown or resynthesis, rather than with the fate of the amino acids liberated in this process (Engel, Schiller and Pentz, 1949).

The fact that glucose administration prevented the expected effect of adrenocortical extract indicates that the glucose effect is exerted peripherally, rather than in the liver. This is consistent with the observations of Engel, Schiller and Pentz (1949) that no correlation could be observed between the liver glycogen levels and the rate of urea formation in nephrectomized rats. Apparently both the action of adrenocortical extract and the antagonistic effect of glucose occur in the peripheral tissues.

The protein-conserving effect of thyroidectomy in the fasting mouse has been shown to be somewhat different from the effect of adrenalectomy, in that carcass protein is spared after the removal of either the adrenal or thyroid gland, whereas sparing of lymphoid nitrogen occurs only after removal of the adrenal (White and Dougherty, 1947). The effect of adrenal activity therefore appears to be exerted both on the carcass and on the lymphoid tissue, whereas the effect of the thyroid is primarily on the carcass. In the present

42B)⁴, 1 mg. per day in three divided doses for 12 days. (3) Seven young females hypophysectomized for 9 to 14 days and three adult males hypophysectomized⁵ for 30 days. (4) Twelve hypophysectomized mice were injected with adrenocorticotrophin; four receiving 0.6 mgm. daily for 6 days; four 0.75 mgm. daily for 8 days; and four, 1 mg. per day for 12 days. (5) Four hypophysectomized mice were injected subcutaneously with pituitary gonadotrophin (Armour—lot FW-234); two receiving 1 mg. per day for 7 days and two receiving 1.5 mg. per day for 13 days. (6) Nine mice were adrenalectomized for 9 to 10 days and maintained on 0.9% sodium chloride in their drinking water.

Twelve rats of the Sprague-Dawley strain were used.⁶ Of these, six were hypophysectomized when 25 days old and maintained for 106 days on a high carbohydrate purified ration (Ration 100 of Shaw and Greep, '49). Six were unoperated controls on the same purified ration for 120 days. Four rats were adrenalectomized and received 0.9% NaCl in their drinking water.

Two adult female rats of the Long Evans strain were started on thiouracil, 0.1% in their drinking water one week before parturition and, together with their litters, comprising 14 male and female rats, were kept on thiouracil for 10 to 16 weeks.

The interscapular brown fat of all the above animals was fixed in 10% neutral formalin and studied in 5 micra paraffin sections stained with hematoxylin and eosin or with eosin and methylene blue using Putt's ('48) modification for formalin-fixed material. In a few instances frozen sections were also made and stained by Baker's ('46) acid haematein method for phospholipins.

INTRODUCTORY REMARKS

The interscapular brown adipose tissue of rodents is readily distinguished from the overlying subcutaneous fat both grossly and in histological sections. Brown fat cells are smaller than ordinary fat cells and possess conspicuously more cytoplasm. Instead of a large single droplet these cells contain multiple droplets of lipid. There are usually two or three vacuoles in each cell which are fairly large and several of smaller size (figs. 1 and 5). In extremely obese animals these lipid droplets coalesce, becoming fewer in number and larger in size so that the brown adipose tissue may come to resemble ordinary fat (fig. 3). In extreme inanition, when the lipids have been exhausted from the fat depots, ordinary subcutaneous fat loses its identity, apparently reverting to areolar tissue. On the other hand, the brown adipose tissue under these conditions persists as lobules of epithelioid cells bearing little or no resemblance to connective tissue.

⁴ We wish to thank Dr. Peter H. Forsham of the Peter Bent Brigham Hospital for the supply of adrenocorticotrophin (Armour) and the Armour Company for the pituitary gonadotrophin.

⁵ Hypophysectomy was performed by the parapharyngeal approach. The term "hypophysectomy" is used in this paper to mean that the anterior and intermediate lobes are removed. For details concerning the technique of hypophysectomy and an analysis of the adrenocorticotrophin and pituitary gonadotrophin preparations see paper by Jones, '49.

⁶ We are indebted to Drs. James H. Shaw and Roy O. Greep of the Harvard School of Dental Medicine for the opportunity to study the brown adipose tissue of these rats.

OBSERVATIONS

Effect of hypophysectomy. Following hypophysectomy the cells of the brown fat gradually lost most of their stored lipid. The few large lipid vacuoles found in the normal animal (fig. 1) gave way to a greater number of fat droplets of small size. These in turn became progressively smaller until the cells ultimately appeared devoid of lipid. When reduced to this condition, the brown fat was a compact, highly vascular tissue of polygonal cells with centrally situated, rounded nuclei (figs. 2, 6, and 7). Although the white adipose tissue also underwent considerable fat loss, there is no doubt that it was less specifically affected by hypophysectomy than was the brown fat. Hypophysectomized animals are generally thought to be in poor nutritional condition, and the cytological changes in brown fat after hypophysectomy were similar to those observed during starvation. Nevertheless, it was possible to show that the changes resulting from hypophysectomy cannot be attributed simply to inanition.

Shaw and Greep ('49) recently investigated the relation of diet to the weight, body composition and duration of survival of hypophysectomized rats. It was shown in their study that when fed on rat chow, not more than 10% of hypophysectomized rats survived beyond 45 days. However, when similar rats were maintained on their purified rations (Ration 100 and Ration 150), 60 to 70% survived 88 to 112 days. We were fortunate in having the opportunity to study adipose tissue from both normal and hypophysectomized rats maintained on these purified diets for over 100 days. The rats in both groups were exceptionally fat animals. There were heavy deposits of subcutaneous fat and the brown fat in the interscapular region and along the great vessels in the thorax was unusually abundant. Relatively little difference was noted between the hypophysectomized and intact rats in the abundance or gross appearance of the brown fat. Nevertheless, histological sections revealed striking differences in the microscopic appearance of the brown fat in the two groups. In the control animals, most of the cells contained one or two very large fat vacuoles (fig. 3), while in the hypophysectomized rats the lipid occurred in numerous small droplets (fig. 4). Thus, the characteristic cytological changes in brown adipose tissue which result from hypophysectomy are mitigated but not prevented by maintaining the animals on high caloric purified diets.

Effect of Adrenalectomy. After adrenalectomy in both rats and mice, the brown fat was depleted of stored lipid and underwent alterations in histological appearance similar to those occurring after hypophysectomy (figs. 9 and 10). Schiffer and Wertheimer ('47) have shown that the decrease in fat stores after adrenalectomy is not due to loss of appetite alone, for adrenalectomized rats show a more extensive loss of body fat than pair-fed, sham-operated controls.

Experiments undertaken with mice to test further the relationship of the pituitary and adrenal glands to brown adipose tissue are summarized in Tables 1 and 2. A subjective evaluation was made of the amount of lipid in histological sections of the interscapular fat of the mice and values from 0 to 5 plus were assigned. The brown fat of normal mice (Table 1-A) was rich in stored lipid (fig. 5) and showed relatively little variation.

Administration of adrenocorticotrophic hormone. Administration of adrenocorticotrophic hormone to intact mice (Table 1-B) had little apparent effect upon brown adipose tissue. It appeared to contain the same amount of lipid or perhaps slightly more than it did in untreated controls. In 9 to 14 days after hypophysectomy (Table 2-A), stored lipid was markedly diminished (fig. 6) and after 30 days it was almost entirely lacking (fig. 7). However, the daily injection of adrenocorticotrophic hormone into hypophysectomized mice (Table 2-B) completely prevented these changes and maintained the normal complement of stored lipids (fig. 8). Injection of a pituitary gonadotrophic preparation had no such effect. (Table 2-C). This preparation served as a control for the adrenocorticotrophin because the two extracts contained the same contaminants and differed only in their active principals.

Hence, it is concluded that hypophysectomy produces its effects upon the interscapular fat by eliminating the normal functional stimulus to the adrenal cortex, and adrenal cortical activity is apparently required for the maintenance of the normal histological structure of brown adipose tissue.

Effect of thiouracil. Littrell ('48) reported that administration of thiouracil to rats brought about a marked decrease in size and an increase in the number of fat globules in the cells of brown fat. These findings were entirely confirmed in the present investigation in which 16 rats received 0.1% thiouracil in their drinking water for varying periods up to 4 months. In the brown fat of these animals, the ma-

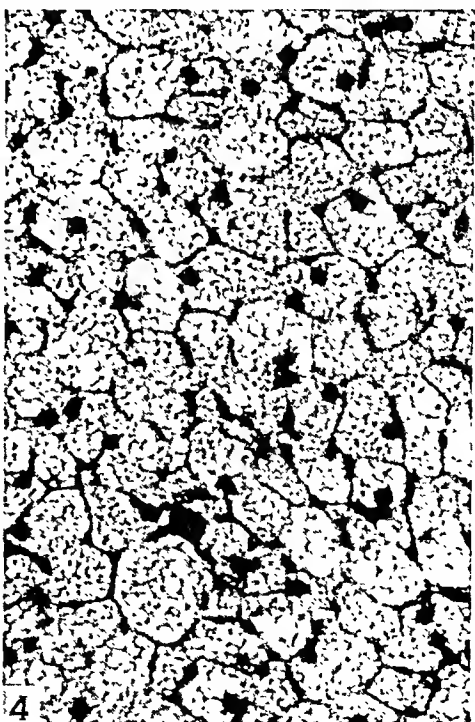
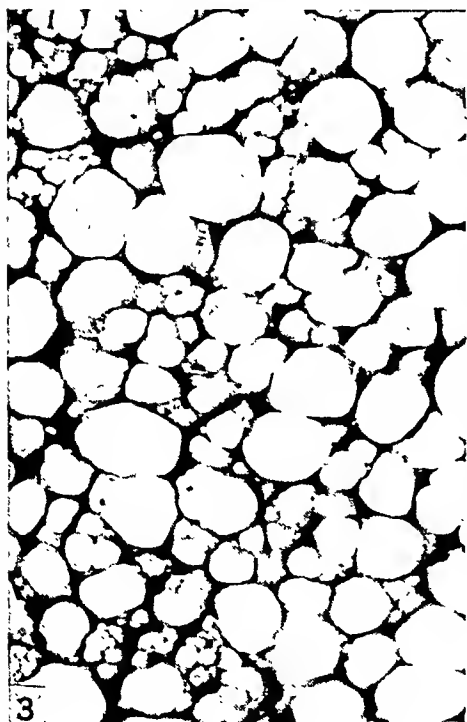
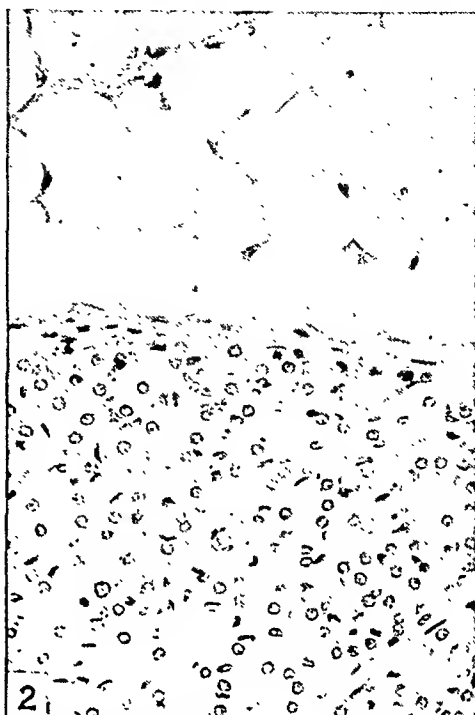
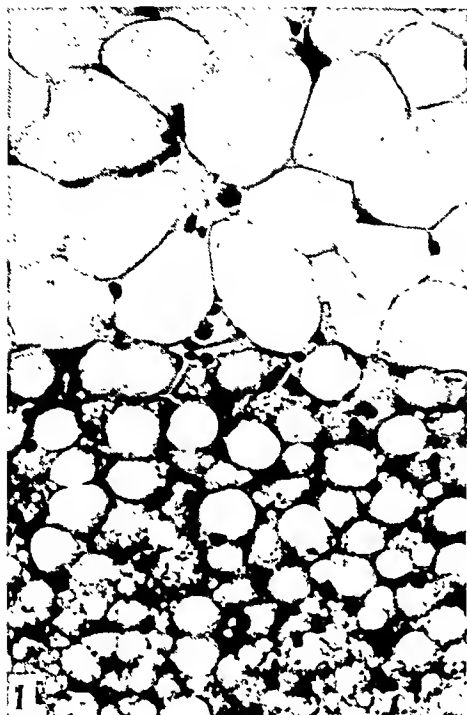
PLATE 1. DESCRIPTION OF FIGURES

FIG. 1. Photomicrograph of interscapular brown fat (below) and adjacent white fat (above) from a normal adult rat fed on rat chow. The cells of the brown adipose tissue are relatively rich in cytoplasm and contain multiple fat droplets. Hematoxylin and eosin. $\times 300$.

FIG. 2. Interscapular fat from a rat hypophysectomized 10 days and fed on rat chow. The fat droplets have disappeared from the brown fat in the lower half of the figure while the overlying white fat has retained its normal appearance. Hematoxylin and eosin. $\times 290$.

FIG. 3. Brown fat of a normal rat fed on a high carbohydrate purified diet (Ration 100) for 125 days. Lipid droplets in many of the cells have coalesced to form single large fat vacuoles, so that the brown fat resembles white fat. Eosin and methylene blue. $\times 385$.

FIG. 4. Brown fat of an hypophysectomized rat maintained for 106 days on the high carbohydrate purified diet. Lipid droplets are numerous but very small, compared to those in the brown fat of the normal animal on same diet (fig. 3). Eosin and methylene blue. $\times 385$.



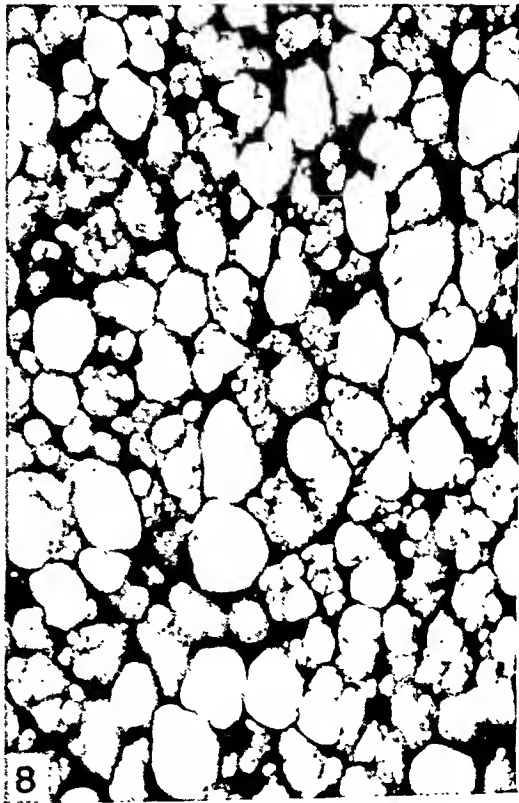
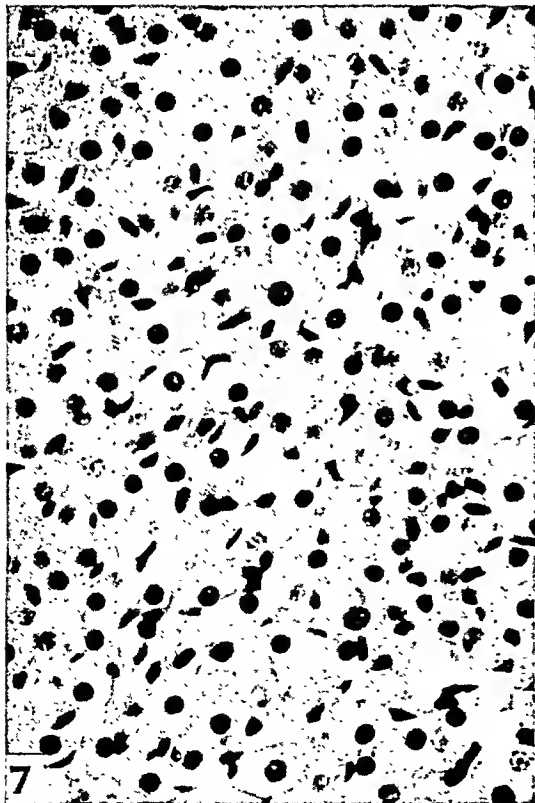
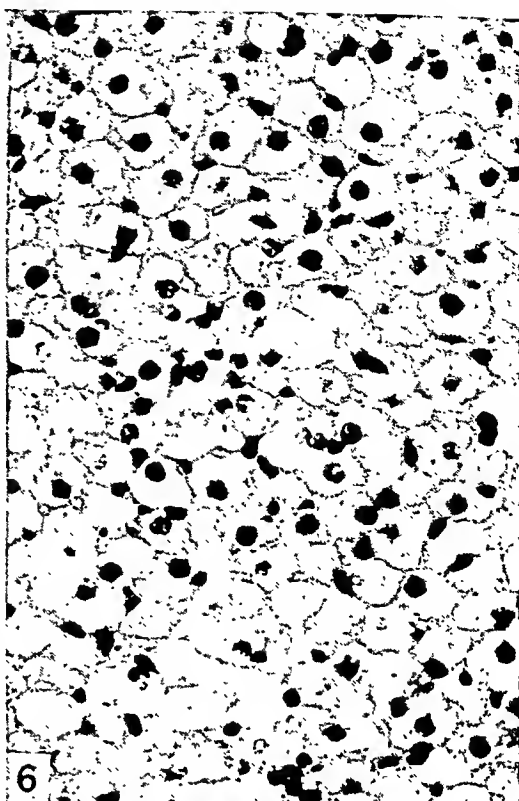
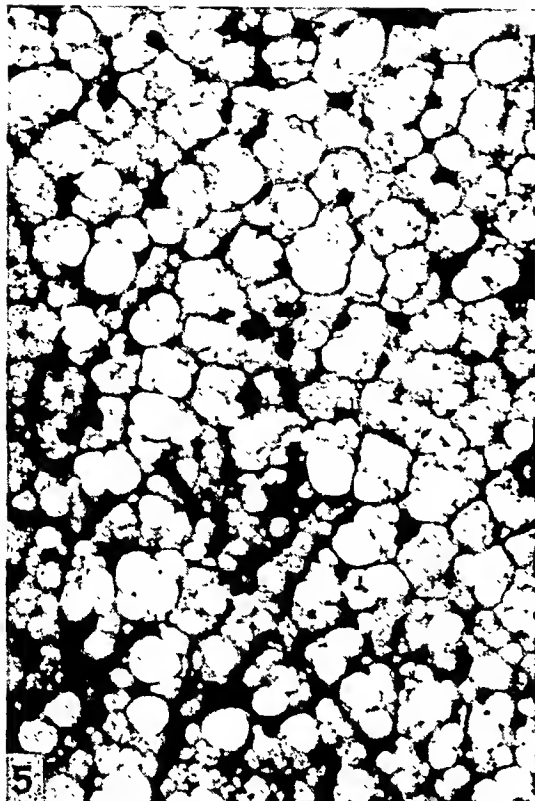


TABLE I
A. Normal mice

Number	Age in days	Days post-op.	Treatment	Fat content interscapular fat
II/9 # 9	55-60	—	—	+++++
7	55-60	—	—	+++++
6	55-60	—	—	+++++
5	55-60	—	—	+++++
32	55-60	—	—	+++++
34	55-60	—	—	+++++
40	55-60	—	—	+++++
16	47	—	—	+++++
20	47	—	—	+++++
B. Normal mice injected with adrenocorticotrophic hormone (ACTH)				
II/9 #26	55-60	—	1.0 mg.	+++++
37	55-60	—	12 days	+++++
29	55-60	—	12 days	+++++
30	55-60	—	12 days	+++++
38	55-60	—	12 days	+++++
33	55-60	—	12 days	+++++
36	55-60	—	12 days	+++++

majority of the cells gradually lost their large fat vacuoles and took on a foamy appearance owing to the presence of large numbers of fine lipid droplets in their cytoplasm (fig. 13). In some animals very little lipid remained in the brown fat while the white fat appeared scarcely affected and retained its normal histological appearance (fig. 15). Scattered through the brown fat were cells which were exceptional in that they contained a single enormous fat vacuole (figs. 12, 13 and 14). These unilocular fat cells occurring singly or in small groups, were especially abundant at the periphery of the lobules and tended to be more numerous in older rats on thiouracil than in young animals. It is impossible to state whether these were atypical brown fat cells or whether they represent an infiltration of the brown adipose tissue by ordinary fat cells.

The cytological changes in brown adipose tissue resulting from thiouracil administration resembled those which follow hypophysectomy and adrenalectomy. They tended, however, to be less severe and took much longer to develop. Approximately three months on thioura-

PLATE 2. DESCRIPTION OF FIGURES

FIG. 5. Interscapular brown fat of a normal mouse is rich in stored lipid (4 plus). Eosin and methylene blue. $\times 385$.

FIG. 6. Brown adipose tissue from a mouse hypophysectomized 9 days. Lipid vacuoles are still present but very much reduced in size. (2 plus). Eosin and methylene blue. $\times 385$.

FIG. 7. Brown fat of a mouse hypophysectomized 14 days. The cells appear to be devoid of lipid droplets. Eosin and methylene blue. $\times 385$.

FIG. 8. Interscapular fat of a mouse hypophysectomized 14 days but injected daily for 12 days with adrenocorticotrophic hormones. The cells have retained their normal complement of stored lipids (5 plus). Eosin and methylene blue. $\times 385$.

TABLE 2
A. Hypophysectomized mice

Number	Age in days	Days post-op.	Treatment	Fat content interscapular fat
II/9 #22	55-60	14	—	++
53	55-60	14	—	+++
II/4 12	47	9	—	+
13	47	9	—	+
II/23 2	50	10	—	0
3	50	10	—	0
8	50	10	—	++
II/Sp 1	adult	30	—	0
2	adult	30	—	0
3	adult	30	—	0

B. Hypophysectomized mice injected with adrenocorticotrophic hormone

II/4 #1	47	9	.6 mg. 6 days	++++
3	47	9	.6 mg. 6 days	++++
9	47	9	.6 mg. 6 days	+++++
14	47	9	.6 mg. 6 days	+++++
II/9 3	55-60	14	1.0 mg. 12 days	++++
14	55-60	14	1.0 mg. 12 days	+++++
20	55-60	14	1.0 mg. 12 days	+++++
27	55-60	14	1.0 mg. 12 days	+++++
II/23 1	50	10	.75 mg. 8 days	++++
2	50	10	.75 mg. 8 days	++
3	50	10	.75 mg. 8 days	+++
10	50	10	.75 mg. 8 days	++++

C. Hypophysectomized mice injected with pituitary gonadotropin

II/4 #2	47	9	1 mg. 7 days	+
2	47	9	1 mg. 7 days	+
II/21 2	60	14	1.5 mg. 13 days	+
5	60	14	1.5 mg. 13 days	0

oil were required to produce changes of the same degree as those found within ten days after adrenalectomy.

DISCUSSION

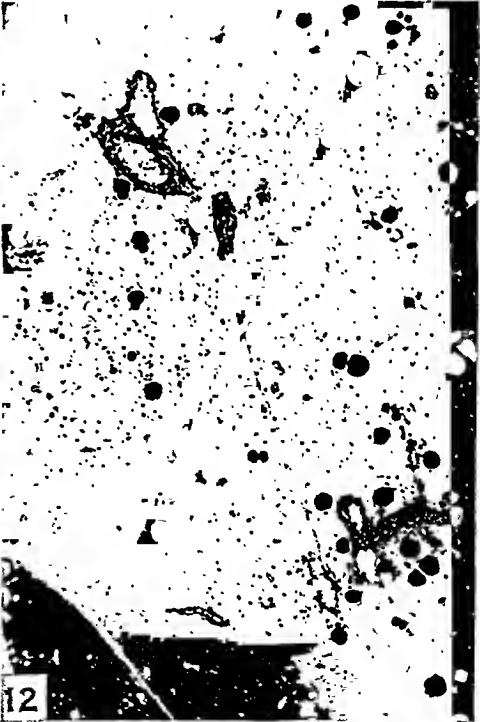
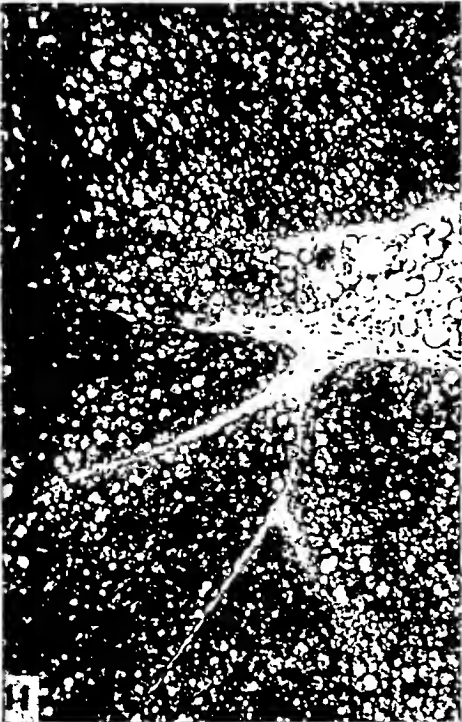
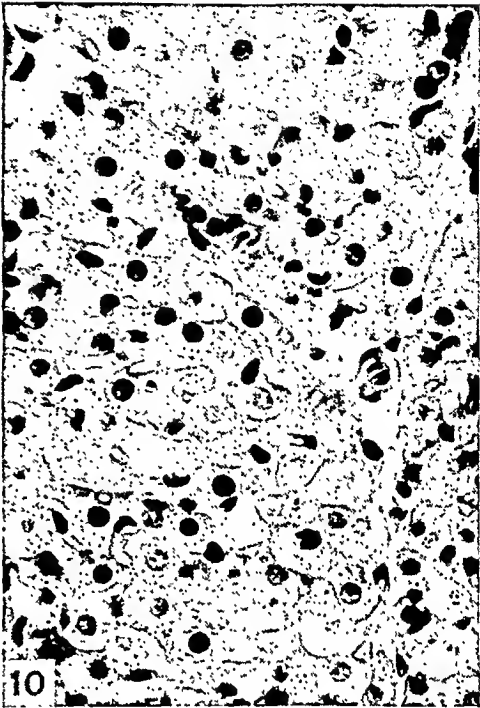
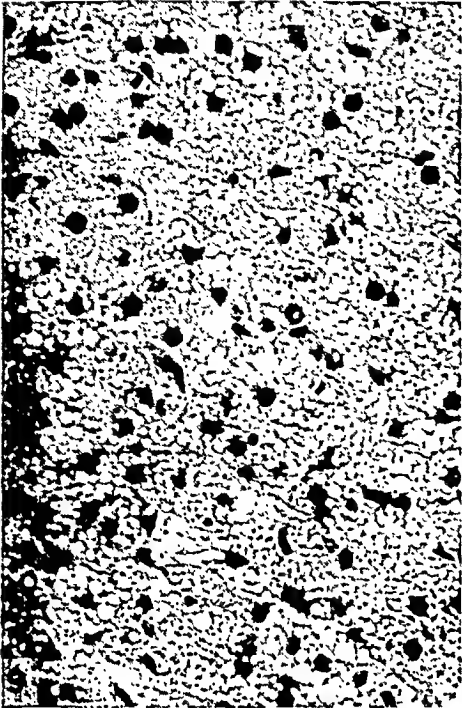
The observations reported here suggest that diminished adrenal cortical function, whether as a result of adrenalectomy or hypophysectomy, is followed by a depletion of the fat depots. This loss of lipid appears to be greater in brown fat than in ordinary adipose

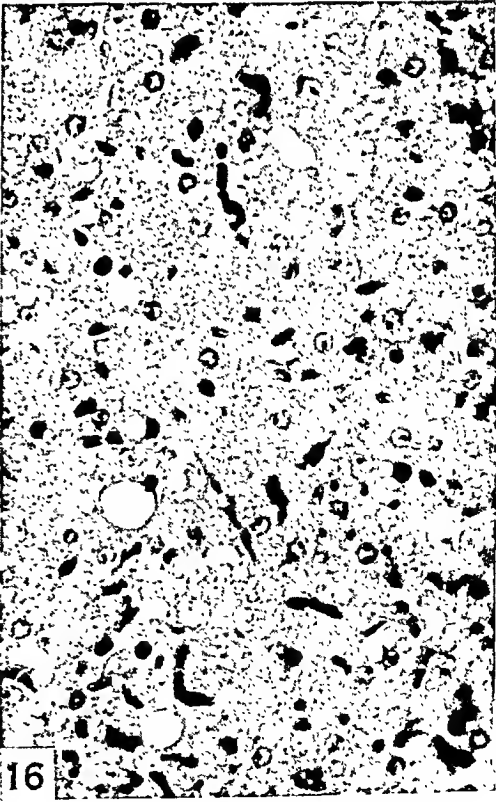
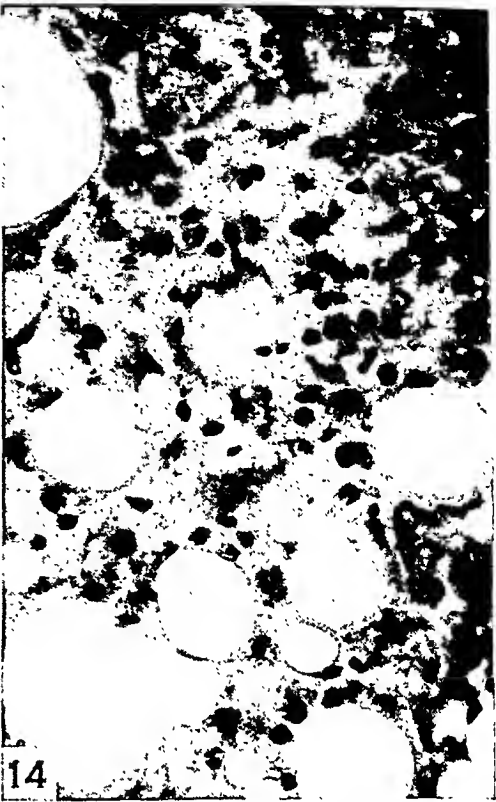
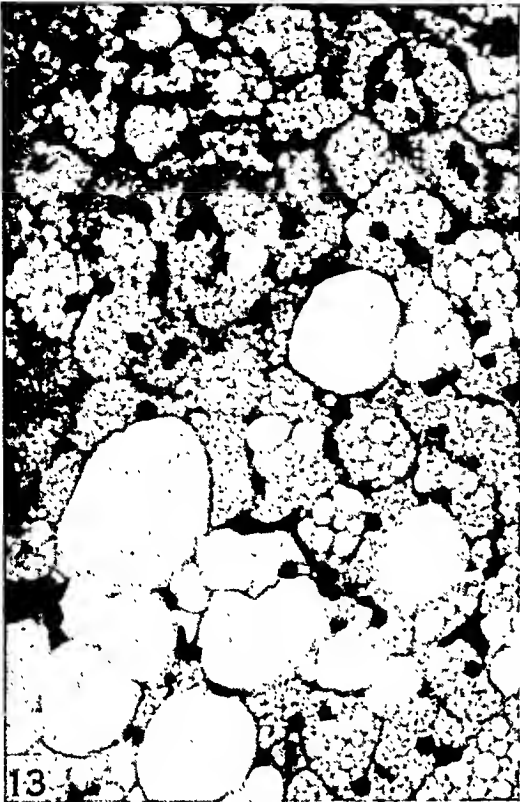
PLATE 3. DESCRIPTION OF FIGURES

FIG. 9. Brown fat of a mouse adrenalectomized for 10 days. Numerous lipid droplets persist but they are very much reduced in size. Eosin and methylene blue. $\times 385$.

FIG. 10. Brown adipose tissue of a rat adrenalectomized for 10 days. Lipid droplets have nearly disappeared. Hematoxylin and eosin. $\times 500$.

FIGS. 11 and 12. Frozen sections of interscapular fat stained by Baker's acid haematein method for phospholipids. $\times 95$. In the normal rat (fig. 11) the cytoplasm of the fat cells stains very intensely because it is rich in phospholipid. The neutral fat appears as colorless vacuoles. In the thiouracil treated rat (fig. 12) phospholipid is still abundant but the bulk of the neutral fat is gone. Scattered through the lobules are certain exceptional cells which contain a single very large fat droplet.





tissue. It is not possible to state whether the loss of adrenal cortical activity affects these changes through impaired fat absorption from the alimentary tract, diminished fat deposition in adipose tissue or interference with fat mobilization and transport. Or, on the other hand whether the reduction of body fat reserves is an indirect result of the effects of adrenalectomy on carbohydrate utilization.

Decreased intestinal absorption of fat after adrenalectomy was reported by Laszt and Verzar ('36) but this has not been confirmed. Indeed, subsequent studies have shown that adrenalectomy does not influence the absorption of fat in rats, provided the intake of food is adequate and the animals are maintained with sodium chloride in the drinking water (Clark and Week, '39). Neither is there any good evidence that the rate of deposition of absorbed dietary fat is reduced by adrenalectomy. Trauer experiments reveal that adrenalectomized rats are capable of incorporating elaidic acid into their depot fat as rapidly as controls (Schiffer and Wertheimer, '47). Nevertheless, a high fat diet does not prevent extensive loss of body fat after adrenalectomy. Experimental procedures which result in accumulation of fat in the livers of normal animals fail to do so in the absence of adrenals. This has been cited as evidence of a disturbance of fat mobilization after adrenalectomy. If this interpretation were correct, the fat depots of adrenalectomized animals would be expected to retain their stored lipid. Experience, however, shows that they do not. Hence, the available experimental evidence seems to indicate that the adrenals do not affect depot fat by any influence on fat absorption, deposition, or mobilization.

Baker *et al.*, ('48) reported that injection of adrenocorticotrophin into normal rats causes a deposition of fat in the liver. This lipotropic effect of the hormone was found to be still more marked if the animals were fed a high carbohydrate diet. It was suggested that adrenocorticotrophin, through its stimulation of the adrenal cortex brought about an increased capacity of the liver to utilize carbohydrate and convert it to fat. It is possible that the influence of the adrenals on the fat depots may largely be secondary to its role in carbohydrate metabolism and fat synthesis.

There is now considerable experimental evidence that the syn-

PLATE 4. DESCRIPTION OF FIGURES

FIG. 13. Interscapular brown fat of an adult rat given thiouracil for three and one half months. The lipid droplets in most of the cells are more numerous than normal but very small. A few cells contain instead a single very large fat droplet. Eosin and methylene blue. $\times 385$.

FIG. 14. Brown adipose tissue from an adult rat which received thiouracil 3 months. Occasional unilocular fat cells are found, but the majority of the cells appear to lack lipid vacuoles. Hematoxylin and eosin. $\times 385$.

FIGS. 15 and 16. Interscapular fat from two young rats on thiouracil for three and one half months. The brown fat contains very little lipid while the area of white fat shown in the upper half of fig. 15 appears normal. Eosin and methylene blue. $\times 385$.

thesis of fat from carbohydrate may take place to some extent in adipose tissue as well as in the liver (Wertheimer and Shapiro, '48). Insulin administration and other experimental procedures which favor the rapid conversion of dietary carbohydrate to depot fat also cause deposition of rather large amounts of glycogen in adipose tissue, particularly in the brown fat (Wertheimer, '45; Fawcett, '48). It has been shown that the breakdown of this glycogen in adipose tissue yields very little glucose (Mirski, '42). It has been suggested, therefore, that the glycogen is degraded to smaller units which may be used by the fat cells for the synthesis of fatty acids (Renold, Marble and Fawcett, '49). Minimal amounts of glycogen are found in the fat cells of adrenalectomized rats under conditions which normally cause marked glycogen deposition (Tuerkischer and Wertheimer, '42). Hence, adrenalectomy may affect the fat depots indirectly by impairing glycogen deposition in the liver and in the adipose tissues, thus interfering with both hepatic and extra-hepatic synthesis of fat from carbohydrate. At best, this explanation would account for only part of the loss of body fat which occurs after adrenalectomy. It leaves entirely unexplained two of Schiffer and Wertheimer's findings, namely the fact that a high fat diet does not prevent fat loss after adrenalectomy and that desoxycorticosterone does prevent it, as well as whole cortical extract. Certainly in the present state of our knowledge concerning the physiology of adipose tissue and the metabolic functions of the endocrine system, we can do little more than speculate as to the mechanism by which the adrenals affect the fat reserves in adipose tissue.

The results of thiouracil feeding on the cytology of brown adipose tissue are in accord with the findings of MacKay and Sherrill ('41) that hypothyroidism in the rat results in a marked reduction in the amount of body fat. This effect may be mediated by the adrenals via the pituitary. Deane and Greep ('47) have demonstrated that in rats fed thiouracil, the zona fasciculata of the adrenal cortex gradually atrophies and shows changes indicative of loss of function. The slower development of the changes in the brown fat of rats on thiouracil may be dependent on the gradual decline of adrenal cortical function which is associated with hypothyroidism.

SUMMARY

In rats and mice after hypophysectomy or adrenalectomy the brown adipose tissue rapidly becomes depleted of its stored lipids. The fat droplets in the cells are transiently increased in number while, at the same time, they are gradually reduced in size. The brown fat is finally reduced to lobules of coarsely granular epithelioid cells which appear to contain little or no lipid. Daily injections of adrenocorticotrophin prevent the development of these cytological changes in the brown fat of hypophysectomized mice. It is concluded,

therefore, that the maintenance of the normal complement of lipid in brown adipose tissue depends upon the functional integrity of the adrenal cortex. The pituitary apparently exercises its effects upon this tissue through its regulation of adrenal cortical activity.

Prolonged ingestion of thiouracil by rats results in histological alterations in their brown fat which are similar to those seen after hypophysectomy or adrenalectomy. These changes develop slowly and may depend upon the gradual atrophy of the adrenal cortex which results from chronic administration of thiouracil.

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NOTES AND COMMENTS

HYALURONIDASE CONTENT IN TESTES FROM RATS TREATED WITH TESTOSTERONE PROPIONATE^{1,2}

McClean and Rowlands (1942) showed that hyaluronidase was capable of dispersing the corona radiata cells surrounding the egg without causing damage to it. The hyaluronidase content of the semen is proportional to the number of spermatozoa (Eichenberger, 1945; Werthessen *et al.*, 1945; Kurzrok *et al.*, 1946; Sherber *et al.*, 1948; Riisfeldt, 1949 a), and it cannot be questioned that it plays a decisive part in the physiology of fertilization. Since the hyaluronidase content of the semen is so closely related to the number of spermatozoa, its formation in the testes must be associated with the spermatogenesis, but so far we have not obtained a detailed knowledge of the process of its formation during the spermatogenesis.

TABLE 1. HYALURONIDASE ACTIVITY IN RAT TESTES FOLLOWING INJECTION OF TESTOSTERONE-PROPIONATE (PERANDREN). THE RATS ARE 3 MONTHS OLD

	Dosage of perandren	No. of rats	Hyaluronidase per gm. wet tissue (V.R.U.)	
			Range	Mean value
Treated for 2 weeks	$\frac{1}{4}$ mg. twice a week	5	230-265	254
	$\frac{1}{2}$ mg. twice a week	5	242-286	268
	1 mg. twice a week	5	235-316	275
	2 mg. twice a week	5	258-354	318
Treated for 4 weeks	$\frac{1}{4}$ mg. twice a week	5	310-362	344
	$\frac{1}{2}$ mg. twice a week	5	304-378	348
	1 mg. twice a week	5	338-490	444
	2 mg. twice a week	5	336-472	432
	No treatment	15	164-210	186

While Sprunt *et al.* (1939) found only negligible amounts of hyaluronidase in testes from immature and cryptorchid rats, Leonard *et al.* (1948) were able to demonstrate that the formation began before the animals were full-grown, and Riisfeldt (1949 b) showed that it was possible to demonstrate the presence of hyaluronidase simultaneously with the appearance of spermatocytes, and that the amount of hyaluronidase increased with the number of spermatocytes and the further progress of the spermatogenesis.

The object of the present study was to examine whether the hyaluronidase content of the testes is affected by hormones, and in the experiments described testosterone propionate (Perandren Merck) was used because of its effect on the epithelium of the testes. However, some diversity of opinion

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¹ Aided by a grant from Kong Christian den Tiendes Fond.

² Testosterone propionate in the form of Perandren has been placed at my disposal by CIBA, Ltd.

exists as to this effect, which also depends on the age of the animal and the dosage.

Rubinstein and Kurland (1941) found an increased proliferation of the epithelium after administration of small doses of testosterone propionate, while degeneration occurred after injection of doses of 2.5 mg. daily.

In the present study 55 Wistar rats were used, 15 of which served as controls. The rats were 3 months old. The dosage of testosterone propionate is seen from Table 1.

TECHNIQUE

From each rat testis a piece of tissue was removed for histologic examination when the treatment was concluded, and the remaining tissue was used for the viscosimetric determination of the hyaluronidase content. A detailed description of the technique of viscosimetric assay of the hyaluronidase content of testicular tissue has been given in a previous publication (Riisfeldt, 1949 c). Viscosity reducing units (V.R.U.) were used in the measurement of the hyaluronidase.

RESULTS

With the doses of testosterone propionate used an increased proliferation of the germinal epithelium was found in all cases; there was an increased number of mitoses and spermatocytes, while no changes in Leydig's cells were demonstrable. In testes from rats which had been treated for four weeks the proliferation was most intense.

From Table 1 it appears that all the rats which had been treated with testosterone propionate for two weeks had a higher amount of hyaluronidase in their testes than the average of the controls, even though the values for some testes were lower than the maximum values among the controls. Rats treated for four weeks showed a hyaluronidase content, which was considerably higher than that of the controls and that of rats treated for two weeks.

The increased proliferation, which involves a larger number of cells in the testes, thus conditions a higher content of hyaluronidase per gram tissue. It is impossible to decide whether the content of the individual cells is higher than that of untreated cells. However, the increased number of cells alone is sufficient to account for the higher content of hyaluronidase, and it may reasonably be supposed that the explanation is to be found here.

SUMMARY

It is shown that the hyaluronidase content in testes from rats treated with testosterone propionate is higher than that of testes from untreated rats.

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METABOLIC EFFECTS OF THYROXINE INJECTED INTO NORMAL, THIOURACIL-TREATED AND THYROIDECTOMIZED RATS¹

Since the rat has classically been considered an animal remarkably refractory to thyroxine, this report is being written to point out the great change in sensitivity produced by thyroidectomy. Credit must be given Meyer and Wertz (1939) for making similar observations 10 years ago, using orally administered thyroxine, but their results have long been ignored, possibly because of the claim of 25 to 30 fold increases in responsiveness.

Young adult male albino rats of the Sprague-Dawley strain, weighing 280-350 grams, were used in this study. They were placed on the limited feeding regime used in this laboratory in which food intake is restricted to 5 hours per day, resulting in an essentially post-absorptive animal which was available each day for metabolic study. Such rats were carefully trained in the Benedict (1930) multiple-chamber, closed-circuit apparatus for determination of oxygen consumption. When thiouracil² was administered, 2 gm. of the drug were thoroughly mixed into each kg. of powdered Rockland Rat Diet. DL-Thyroxine³ was injected subcutaneously in an alkaline isotonic solution.

A single dose of 6.12 mg. thyroxine (equivalent to 4.0 mg. iodine) per kg. body weight caused a 30% increase 24 to 48 hours later in the metabolic rate of normal rats, as can be seen from Figure 1. A week later, the BMR had returned to the control level. With animals administered thiouracil for 3-5 weeks, the extent of the metabolic response was approximately the same as that shown by the normals, as previously reported for desiccated thyroid substance (Barker, 1945). However, thyroidectomized animals responded with a much greater metabolic rise which lasted 3 weeks instead of a few days. Figure 1 shows these comparisons graphically. The injection of 612 μ g. per kg. per day for 5 days gave similar results in that the thyroidectomized animals exhibited a metabolic rise of 70% which required 13 days after the last injection to return to the pre-injection level as compared to an elevation of 44% in normals which had disappeared in 6 days.

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¹ This investigation was supported by a grant from the Division of Research Grants and Fellowships, National Institutes of Health, U. S. Public Health Service.

² Thiouracil was furnished by Dr. Stanton M. Hardy of the Lederle Laboratories, Pearl River, N. Y.

³ DL-thyroxine was made available by Dr. K. W. Thompson of Organon, Inc., Orange, N. J.

One one-hundredth of the original dose, or 61 μ g. thyroxine per kg. body weight, injected per day into 4 thyroidectomized animals produced by the 5th day an increase of 32 cc. O_2 per 100 gm. per hr., 60% as much response as that with the 612 μ g. dose. When the amount administered was next decreased to 12 μ g. thyroxine per kg. per day, the O_2 consumption of 12 thyroidectomized animals was increased by 18 cc. per hr. (Figure 2). After experiments testing thyroxine analogues for their thyroxine-inhibiting effects had been conducted on these animals, they were continued on 12 μ g. thyroxine, allowing the BMR's to return to their previous elevated values. The hormone was then withdrawn and a prompt fall resulted in every instance.

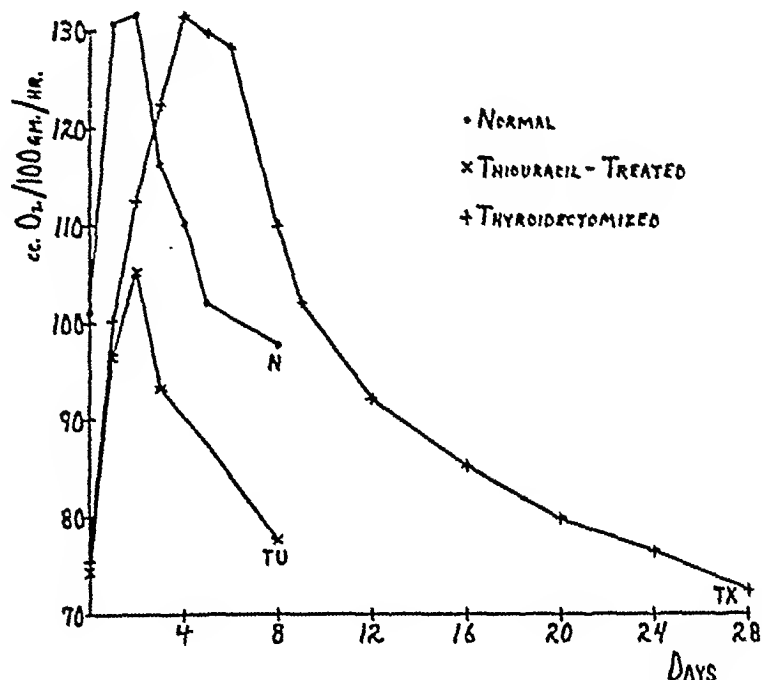


FIG. 1. Metabolic response of normal, thiouracil-treated and thyroidectomized rats to a single injection of 6.12 μ g. DI-thyroxine.

Three of 4 animals tested with 6 μ g. thyroxine showed an average increase of 9 cc. O_2 by 12 days which was well maintained for another 5 days on treatment. This set also reverted to the pre-treatment hypometabolic status when thyroxine injections were stopped. Further work is in progress extending the analysis to still lower doses than 6 μ g. per kg. per day. Although not shown, normal animals have been given 12 μ g. and thiouracil-treated 6 μ g. per kilo per day for three weeks without changing the metabolic rate of either group.

Various workers have estimated the amount of thyroxine secreted at normal temperatures by the rat to be about 35 to 100 μ g. of DI-thyroxine per kg. per day on the basis of regression of thyroid gland size in thiouracil-treated animals (Dempsey and Astwood, 1943; Schultze and Turner, 1945; Frieden and Winzler, 1948). From our data, not more than 20 μ g. of DI-

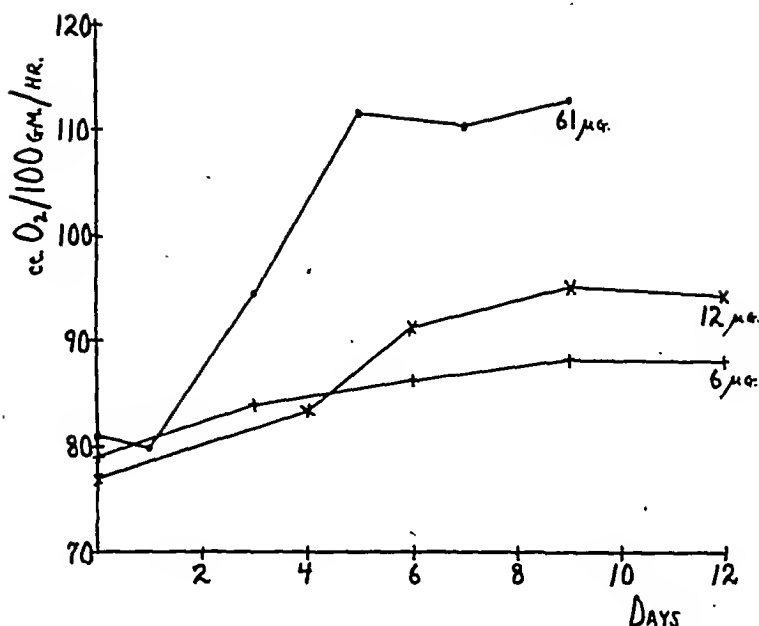


FIG. 2. Metabolic response of thyroidectomized rats to small doses of DL-thyroxine. The amounts shown were injected per kg. per day.

thyroxine would be required per kg. per day to return the metabolic rate of thyroidectomized rats to normal, one-half to one-fifth of the estimates quoted above.

The clear differentiation noted in these results between normal and thiouracil-treated rats on the one hand and thyroidectomized on the other further emphasizes the growing realization that the effects of the drug extend beyond the thyroid gland (cf. Borell and Holmgren, 1948). The agranulocytosis seen in some patients treated with thiouracils probably represents such a depression of an extra-thyroidal tissue, the bone marrow.

SUMMARY

The thyroidectomized rat has been found to give a metabolic response to a daily injection of 6 µg. DL-thyroxine per kilogram of body weight, an amount inadequate to produce any increase in the oxygen consumption of normal or thiouracil-treated animals. The decreased metabolic rate of thyroidectomized rats is returned nearly to normal by 12 µg. DL-thyroxine per kg. per day. A chronic mammalian preparation of high sensitivity is thus made available for many aspects of thyroid study.

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DIABETOGENIC ACTION OF PURIFIED ANTERIOR PITUITARY HORMONES

This communication deals with the diabetogenic action of three of the anterior pituitary hormones, namely growth hormone, adrenocorticotrophic hormone (ACTH) and prolactin. Previous work has indicated that purified growth hormone is diabetogenic (Marx *et al.*, 1943; Houssay, 1945).

The growth hormone, which had been prepared by Armour and Co.¹ (lots #8KR32 and #3PKR3) exhibited a purity, as judged by the electrophoretic diagram, of approximately 84 per cent of a single component which had a mobility comparable to that of purified growth hormone. Assay of the material in a 60-70 gram hypophysectomized rat showed that a 50 gamma dose per day caused a body increment of 1.0 gram per day. The ACTH, which was also supplied by the Armour Laboratories (lot #45-A-4), had been prepared by a modification of the Sayers method. Biological assay on hypophysectomized rats, making use of the ascorbic acid depletion method of Sayers *et al.* (1948), showed a potency of 96 per cent. The amounts of contaminating hormones such as prolactin, growth, thyrotrophic and gonadotrophic hormones, were negligible. The preparation exhibited 0.4 units per mg. of posterior pituitary activity. The prolactin, which was supplied by E. R. Squibb and Sons² (lot #71713), showed a potency of 24.2 units per mg. The preparation did not contain an amount of growth hormone sufficient to increase the body weight of 4 hypophysectomized rats which were injected with 5 mg. daily (80 mg. total) over a period of 17 days; nor of 2 hypophysectomized rats which received 10 mg. daily (110 mg. total) for 11 days. It did contain ACTH since the average weight of the adrenal glands in the group which received the 80 mg. total dose was 28 mg.; the rats which received the 110 mg. amounts had 31 mg. adrenals, while the uninjected controls had 20 mg. adrenals. By the Sayers assay method this lot of prolactin was found to contain 10-30% ACTH. According to the rooster blood pressure method, 1 mg. of the preparation contained 0.008 international units of posterior pituitary activity. The gonadotrophic and thyrotrophic hormones were present in negligible amounts.

Partial pancreatectomy was performed on dogs, cats and rats. It has been found that a decrease in the mass of pancreatic tissue greatly increases the sensitivity of the diabetogenic action of the pituitary. In the dogs 83-87% of the pancreas was removed, in the cats 73-75%, and in the rats 95%. Batrachians were also used; in these the pancreas and the anterior pituitary were removed in their entirety. All of the mammals showed a

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¹ We wish to thank Dr. J. R. Mote of Armour and Co for supplying us with the growth hormone and the adrenocorticotrophic hormone.

² We wish to thank Dr. Robert Bates of E. R. Squibb and Sons for supplying us with the prolactin.

normal blood sugar before the injections were started. The dogs were given 50 mg. of hormone per kg. per day for 4 days, the cats 25 mg. per kg. per day for 3 days, and the rats 25 mg. per kg. per day for 3 days. These injections were made by the intra-peritoneal route. Fasting blood sugar determinations were made daily during this period. The batrachians received 1.0 mg. per day. The injection was given subcutaneously immediately after the anterior pituitary and the pancreas had been removed. The blood sugar was determined 24 hours later.

TABLE 1. DIABETOGENIC ACTION OF PURIFIED ANTERIOR PITUITARY HORMONES

Animal No.	Highest fasting blood sugar during injection period*		
	Growth hormone	Prolactin	ACTH
	mg. %	mg. %	mg. %
Dog #30	336	168	140
Dog #2	296	147	86
Dog #5	185	134	89
Dog #10	90	133	84
Cat #1	304	97	
Cat #3	278	221	
Cat #6		203	
Cat #7		167	
Cat #9		221	
Rat #1204	158		
Rat #1206	134		
<i>Rana pipiens</i> Injected (av. 10)	144	70	163
Control (av. 10)	78		
<i>Bufo arenarum</i> Injected (av. 30)	130	108	121
Control (av. 30)	81		

* Fasting blood sugar before injections was normal in every case.

The results of this study are shown in Table 1. The growth hormone produced hyperglycemia, glycosuria and polyuria in 3 of the 4 dogs, in both of the cats so treated, and in all 40 batrachians used.

In dog #30 a permanent diabetes developed as a result of the injections. In addition to the 3 courses of injections as indicated in Table 1, on 3/30/49 and 3/31/49 the injections of growth hormone were repeated. On 4/1/49 its fasting blood sugar was 336 mg.% and from then on until it was sacrificed (9/1/49) its fasting blood sugar averaged 200 mg.%. Clinical evidence of diabetes was apparent. Body weight fell from 20 lbs. at the time of the first injections (2/12/40) to 12 lbs., when the animal was sacrificed. A glucose tolerance test on 8/17/49 in which 50 cc. of 20% glucose were administered orally showed the following: fasting blood sugar, 361 mg.%; $\frac{1}{2}$ hour, 432 mg.%; 1 hour, 576 mg.%; 2 hour, 440 mg.%; 3 hour, 348 mg.%; 4 hour, 327 mg.%; 5 hour, 328 mg.%. During the last month 8 units of protamine zinc insulin were given daily.

Prolactin produced an elevated fasting blood sugar in all 4 dogs injected, in 4 of the 5 cats, and in 60% of the batrachians. In the dogs and cats there was glycosuria and polyuria in addition to the high blood sugar.

ACTH produced hyperglycemia in only one dog of the 4 injected, and in 83 per cent of the batrachians. The experiments with dogs had to be interrupted because of insufficient ACTH.

The diabetogenic action of prolactin could be due to the ACTH since this preparation contained 10-30 per cent of ACTH. But the prolactin effect was more intense than that of the purified ACTH, which had a potency of 96 per cent. It is possible that the hyperglycemic action of the prolactin could be due to a potentiation of ACTH action by prolactin. Another interpretation may be that the effect of the two hormones was additive. The possibility that another diabetogenic agent was present in the prolactin preparation could not be ruled out.

CONCLUSIONS

These experiments indicate that the growth hormone has a diabetogenic action on dogs, cats and batrachians.

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³ We wish to thank Mr. W. H. Good, Mr. Allan Moore and Dr. E. J. Schrenzel for their technical assistance.

NEW BOOK

PHYSIOLOGY OF THE UTERUS. 2nd Edition. S. R. M. Reynolds. Paul B. Hoeber, Inc. New York. xx+611 pp. \$12.50.

This is a book of strong contrasts, combining singular virtues with serious weaknesses. On one hand, it gathers together an enormous mass of information, provides a comprehensive bibliography and offers the only integrated guide to the field. On the other, there are many examples of poor organization, misplaced emphasis and faulty digestion of the data cited.

Examples of contradictory statements and poor organization could be selected almost at random. A few instances will suffice. On page 107, in discussing a possible placental origin of progesterone, the author says "Aside from the fact that a progesterone-like extract has been prepared from human placentas, there is no conclusive evidence to show that such a hormone is secreted, rather than stored, in the placenta, and some workers have failed to find such a substance." Such a statement certainly implies doubt, at least, that the placenta can produce this hormone. Yet, on pp. 117 and 118, the following paragraph is found.

"Immediately preceding labor, or accompanying false labor, the excretion of pregnanediol diminishes markedly. There is, accordingly, much progesterone produced during pregnancy, at times when the ovaries are not essential. It is presumed, therefore, that progesterone is secreted by the placenta (Fig. 19). It was with extracts from this tissue, in fact, that some of the earliest progestational effects were obtained. Excision of the corpus luteum in the very early months of pregnancy, however, leads to cessation of pregnanediol excretion although not if the operation is at a later time."

The trail becomes still more obscure when one notes that figure 19 is captioned "Proof that the human placenta contains progesterone-like substances in late pregnancy," that fig. 20 illustrates that pregnanediol excretion is essentially unaltered after ovariectomy at the 14th week of gestation, and that "The best evidence for the production of progesterone is found, therefore, in the identification of its degradation products, particularly pregnanediol." Moreover, there seems to be no doubt as to the adequacy of the experimental method, since on p. 116 the following statement occurs.

"In the human, and possibly other species as well, estrogen is clearly elaborated by the placenta, since ovariectomy in the human does not, as mentioned already, modify the estrogen-excretion curve, yet immediately after parturition the urinary estrogen level drops to that observed in castrated women."

To sum up, after ovariectomy during pregnancy, estrogen excretion is maintained until term, and this is clear evidence for the secretion of estrogen by the placenta. But with respect to progesterone, an exactly equivalent set of facts leads the author to conclude that the hormone may only be stored, not secreted, by the placenta. The logic is faulty for one of these two propositions.

The bibliography is extensive and valuable, yet its organization leaves something to be desired. It is divided into eight sections, corresponding to a

similar parcellation of 37 chapters. Each section of the bibliography follows its corresponding section in the text. Consequently, the index becomes cumbersome, and finding a particular textual citation from the author index requires several steps. First the appropriate section must be located, then the text must be searched for the assigned reference number. More usable reference systems are well-known.

Despite these criticisms, the book is invaluable to the student of reproduction. It is the only one of its kind, and it contains a vast amount of information. The impression it makes of hasty preparation is regrettable.

E.W.D.

ANNOUNCEMENT OF THE 1950 MEETING OF THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

The Thirty-Second Annual Meeting of The Association for the Study of Internal Secretions will be held at the Sir Francis Drake Hotel, Friday and Saturday, June 23 and 24, 1950, in San Francisco, California.

The Committee on Local Arrangements is comprised of Dr. Hans Lisser, Chairman and Doctors Leslie L. Bennett, Roberto F. Escamilla, Minnie B. Goldberg, Gilbert S. Gordan, Laurance W. Kinsell.

Hotel accommodations will be difficult to secure on short notice; therefore, members are urged to make their reservations at once. All requests must be addressed to: Dr. William Howard Rustad, American Medical Association (Hotel Committee), Room 200, Civic Auditorium, San Francisco, California.

The scientific sessions will be held in the Empire Room of the Sir Francis Drake, and registration will be on the same floor. The annual dinner will be held in the Empire Room on Friday, June 23 at 7:30 P.M., preceded by cocktails at 6:30 P.M.

Those wishing to present papers, which will be limited to ten minutes, should send title and four copies of an abstract of not more than 200 words, to Edward A. Doisy, M.D., St. Louis University School of Medicine, 1402 South Grand Avenue, St. Louis, Missouri, not later than March 1, 1950. *It is imperative that the abstracts be informative and complete with results and conclusions in order that they may be of reference value and suitable for printing in the program and journals of the Association.* Names of non-members who are co-authors must be followed by the words "by invitation," and the principal degree of each author.

Nominations for the Ayerst, McKenna and Harrison Fellowship, the Schering Fellowship in Endocrinology, and the Squibb and Ciba Awards should be made on special application forms which may be obtained from the Secretary-Treasurer, Henry H. Turner, M.D., 1200 North Walker Street, Oklahoma City 3, Oklahoma, and filed with the Secretary not later than March 15, 1950.

Announcing

POSTGRADUATE ASSEMBLY IN ENDOCRINOLOGY INCLUDING DIABETES

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Miami Beach, Florida Roney Plaza Hotel April 3-8, 1950

The faculty will consist of prominent researchers and clinicians in the field of endocrinology and metabolic disorders, gathered from the United States and Canada.

The course will be a practical one of interest and value to the specialist and those in general practice. The program will consist of lectures, clinics and demonstrations. Ample time will be given to questions and answers at the end of each session, and registrants are encouraged to contact members of the faculty for individual discussions.

The Roney Plaza, one of Miami Beach's most delightful hotels, offers special convention rates to members of this assembly. This is an unusual opportunity for you and your family to enjoy a pleasant vacation and for you to participate in a highly instructive program of the latest advances in endocrinology and metabolism.

A fee of \$75 will be charged for the entire course and the attendance will be limited to 100. REGISTRATION WILL BE IN THE ORDER OF CHECKS RECEIVED AND WILL CLOSE ON MARCH 3, 1950. Should there be an insufficient number of applicants to fill the course, the registration fee will be refunded immediately in its full amount.

Application for approval of this course has been made to the Veterans Administration. Veterans should make formal application to their local agencies on the appropriate form (1905e or 1950) as furnished by the V.A.

Please forward application on your letterhead together with check payable to The Association for the Study of Internal Secretions, to Henry H. Turner, M.D., Secretary-Treasurer, 1200 North Walker Street, Oklahoma City 3, Oklahoma, before March 3, 1950. Further information and program will be furnished upon request.

Hotel reservations should be made directly with the Roney Plaza Hotel, Miami Beach, Florida, and the hotel advised that you are attending this Postgraduate Assembly.

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